ABSTRACT OF THESIS

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Title of Thesis CHOLESTEROL OXIDATION IN RAT LIVER

This study is concerned with the cholesterol-7α-hydroxylase enzyme of the rat liver microsomes. The aims of the study were as follows. (1) To establish the relationship of the cholesterol-7α-hydroxylase to the other hydroxylase enzymes of the liver microsomes, with emphasis on the role of the oxygen activating cytochrome P450. (2) To characterise the components of the cholesterol-7α-hydroxylase. (3) To examine the relationship between the formation of non-physiological oxidation products of cholesterol and the peroxidation of unsaturated fatty acids. (4) To investigate the nature of the inhibitor of non-physiological cholesterol 'oxidation' which is present in the heat-stable portion of the rat liver supernatant.

The liver microsomal cholesterol-7α-hydroxylase is inhibited by the presence of carbon monoxide and the inhibition is reversed by light, the most effective wavelength for release of the inhibition being 450 nm. This implicates the microsomal cytochrome P450 in the hydroxylation of cholesterol. Induction of the cholesterol 7α-hydroxylase by interrupting the entero-hepatic circulation of bile acids causes no increase in the amount of cytochrome P450 in the microsomes, nor does it have an effect on the hydroxylation of aniline or aminopyrine, both of which are hydroxylated by P450-dependent reactions. In addition no differences are found in the spectral properties of the cytochrome P450 of microsomes from rats with raised cholesterol-7α-hydroxylase activity, when compared with normal rats. It is concluded therefore that either the cytochrome P450 involved in the cholesterol-7α-hydroxylase is only a small part of the total liver microsomal cytochrome P450, or that the rate-limiting step of the reaction does not reside in the P450.

The rat liver microsomal cholesterol 7α-hydroxylase appears to have as one of its components the NADPH-cytochrome c reductase of the liver microsomes. Interaction between the NADPH-cytochrome c reductase and the cytochrome P450 is necessary for cholesterol 7α-hydroxylase activity.

It was established that cholesterol autoxidation and unsaturated fatty acid peroxidation are similar autocatalytic free radical reactions of the oxidisable components of the microsomes. Both have an absolute requirement for NADPH and oxygen, both are stimulated by ADP and ferrous ions, and both are inhibited by cysteamine, EDTA, cyanide and manganous ions. Cysteamine and EDTA also have the effect of stimulating the production of 7α-hydroxycholesterol at higher concentrations.

Use other side if necessary.

CHOLESTEROL OXIDATION IN RAT LIVER

Alexander Michael Grimwade

Submitted for the degree of Doctor of Philosophy in the University of Edinburgh

October 1971

The heat-treated 105,000 g supernatant of the rat liver has the ability to inhibit the peroxidation of unsaturated fatty acids and the production of non-physiological oxidation products of cholesterol, and at higher concentrations stimulates the production of 7α-hydroxycholesterol. It therefore contains factors which direct cholesterol oxidation towards 7α-hydroxylation and away from peroxidation reactions. The factor(s) (S.F.) in the heat-treated supernatant which inhibit the peroxidation reactions is of small molecular weight, is non-lipid and requires intact thiol groups for its operation. Fractionation by exclusion and ion-exchange chromatography followed by electrophoresis led to the identification of the thiol component as reduced glutathione. Commercial GSH does not have the same effect on cholesterol oxidation and lipid peroxidation as S.F. It is possible that an unidentified second factor which modifies or potentiates the GSH may be present in the S.F. On comparison of the pK of the heat-treated supernatant thiol and commercial GSH, no difference was found.

The S.F. was inactive in the reduction of cholesterol 7α-hydroperoxide to 7α-hydroxycholesterol. GSH in the presence of some metal ions especially manganous ions, has the same effect on cholesterol oxidation as S.F., although neither of these compounds acts in this manner separately. The concentration of manganous ions in the rat liver supernatant is too low for this cooperative action of GSH and Mn^{2+} to be important physiologically.

This thesis has been composed entirely by myself, and all the experimental results, except where otherwise acknowledged are the product of my own work. This study has been part of the research programme of the Medical Research Council's Research Group in Steroid Metabolism under Professor G.S. Boyd.

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My thanks to Professor G.S. Boyd for his expert supervision throughout this study, to Margaret E. Lawson for her help and advice, and to my laboratory colleagues for many valuable discussions.

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Abstract of Thesis.

Cholesterol oxidation in rat liver.

This study is concerned with the cholesterol-7αhydroxylase enzyme of the rat liver microsomes. The aims of the study were as follows. (1) To establish the relationship of the cholesterol-7αhydroxylase to the other hydroxylase enzymes of the liver microsomes, with emphasis on the role of the oxygen activating cytochrome P450. (2) To characterise the components of the cholesterol-7 hydroxylase. (3) To examine the relationship between the formation of non-physiological oxidation products of cholesterol and the peroxidation of unsaturated fatty acids. (4) To investigate the nature of the inhibitor of non-physiological cholesterol 'oxidation' which is present in the heat-stable portion of the rat liver supernatant.

The liver microsomal cholesterol-7αhydroxylase is inhibited by the presence of carbon monoxide and the inhibition is reversed by light, the most effective wavelength for release of the inhibition being 450 nm. This implicates the microsomal cytochrome P450 in the hydroxylation of cholesterol. Induction of the cholesterol 7α-hydroxylase by interrupting the entero-hepatic circulation of bile acids causes no increase in the amount of cytochrome P450 in the microsomes, nor does it have an effect on the hydroxylation of aniline or aminopyrine, both of which are hydroxylated by P450-dependent reactions. In addition no differences are found in the spectral properties of the cytochrome P450 of microsomes from rats with raised cholesterol-7αhydroxylase activity, when compared

with normal rats. It is concluded therefore that either the cytochrome P450 involved in the cholesterol-7α-hydroxylase is only a small part of the total liver microsomal cytochrome P450, or that the rate-limiting step of the reaction does not reside in the P450.

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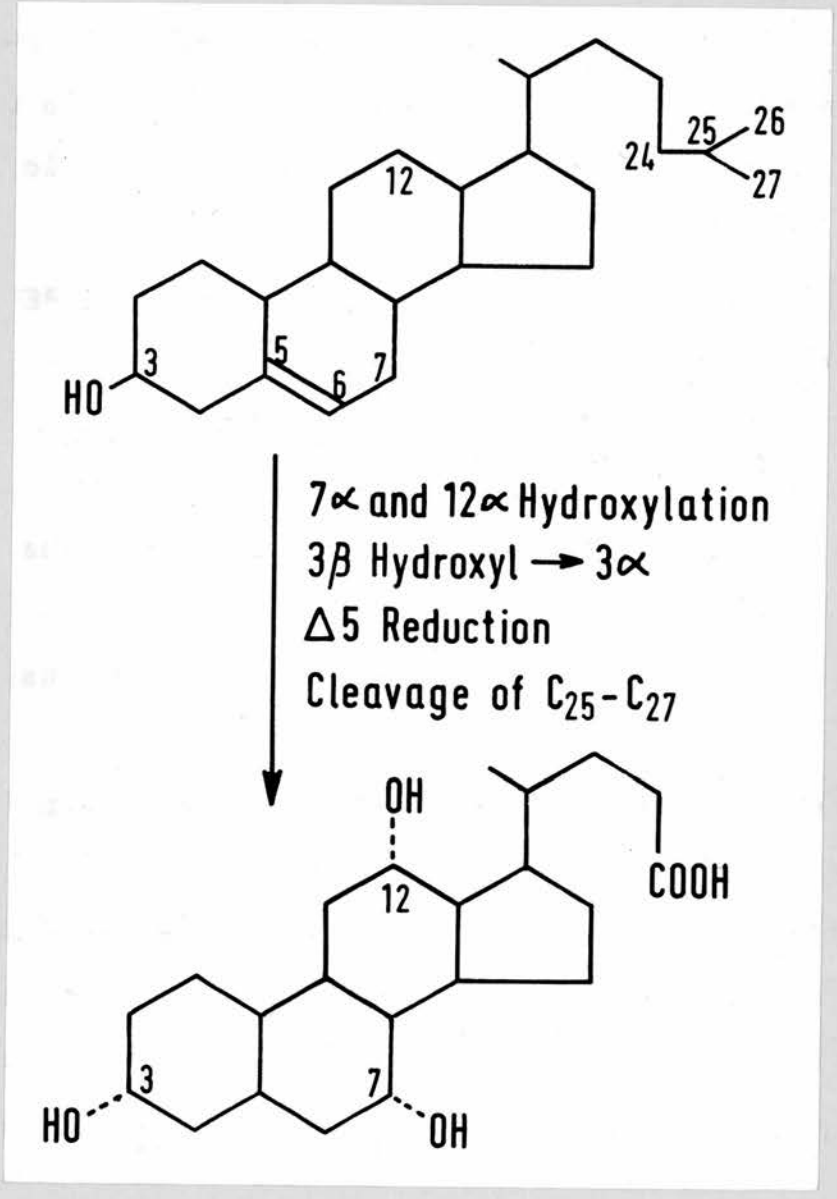

Figure 1. The transformation of cholesterol to cholic acid.

SECTION 1

Introduction

The major catabolic pathway for the cholesterol molecule in mammals is the conversion to bile acids (1). The reactions involved in the conversion of cholesterol to cholic acid are as follows.

(i) Oxidative cleavage of a three carbon fragment from the cholesterol side chain.

(ii) Reduction of the $\Delta 5$ double bond.

(iii) Epimerisation of the 3β hydroxyl group.

(iv) Hydroxylation at the 7α and 12α positions of the sterol nucleus.

Figure 1 shows the transformation of cholesterol to cholic acid.

Linstedt suggested that the initial reaction in the conversion of cholesterol to bile acids in the mammalian liver is the 7α-hydroxylation of cholesterol, (2) and several groups of workers have demonstrated the conversion of cholesterol to cholest-5-en-3β, 7α-diol (7α-hydroxycholesterol) in the liver, and in sub-cellular fractions of the liver (3,4). It has been shown in this laboratory by previous workers that the 18,000 g supernatant of rat liver, fortified with an NADPH generating system will convert cholesterol into a single product, namely, 7α-hydroxycholesterol (5), as shown in Figure 2.

The production of bile acids by the liver can be greatly increased by interrupting the entero-hepatic circulation of bile

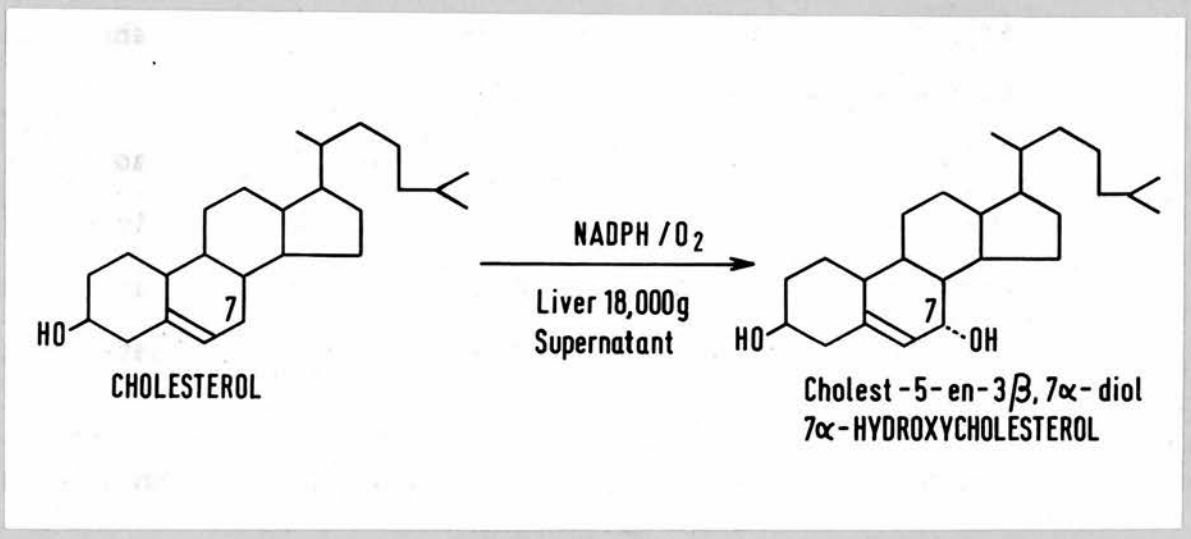

Figure 2. The 7α-hydroxylation of cholesterol.

acids (6). This can be done in two ways. The bile duct can be cannulated and the bile drained externally, or, more simply, cholestyramine, a resin which binds bile acids at the pH of the small intestine, can be administered to the experimental animal in the diet. Both these methods lead to an increased output of bile salts from the liver. If the liver from a bile duct cannulated animal or a cholestyramine fed animal is assayed for cholesterol-7α-hydroxylase activity, it is found that this activity has increased by as much as 5 fold (7).

This, together with the fact that there is no accumulation of the intermediates of bile acid synthesis in the liver, leads to the conclusion that the 7α-hydroxylation of cholesterol is probably the rate limiting step in the conversion of cholesterol to bile acids in the liver.

The cholesterol-7α-hydroxylase enzyme has an absolute requirement for oxygen and NADPH (5). It therefore appears to belong to the category of enzymes known as mixed function oxidases (8). Mixed function oxidases require a supply of reducing equivalents, oxygen, and an oxidisable substrate, which react as follows:

$$RH + H^+ + SH + O_2 \longrightarrow R + SOH + H_2O$$

The mixed function oxidases are therefore characterised by the cleavage of molecular oxygen, one atom being incorporated into the substrate, and the other being reduced to water. Cholesterol is not the only substance known to undergo mixed function oxidation in the liver microsomes. Many lipophilic non-physiological

compounds, often known as 'xenobiotics' (9) are hydroxylated and oxidatively demethylated. These xenobiotics include such compounds as phenobarbital and other barbiturates, benzpyrene and other carcinogens, alkaloids, anaesthetics, insecticides, and dyes.

The enzymology of the hydroxylation of some of these compounds has been extensively studied and, despite the remarkable dissimilarity of the various substrates mentioned above, the hydroxylations of these compounds appear to be mediated by one distinct type of oxygen activating cytochrome (See (10) for review).

Cytochrome P450 was discovered in the liver microsomes in 1958, but it was not until 1963 that its enzymic role was appreciated. This cytochrome was named P450 because of its strong absorption at 450nm in the reduced, carbon monoxide complexed form (11). The hydroxylation of many compounds by the liver microsomes requires reducing equivalents from NADPH, and the reaction can be inhibited by carbon monoxide. P450 is also found in other tissues where mixed function oxidation reactions occur, notably the adrenal cortex mitochondria and microsomes. Estabrook et al. were the first to show that the inhibition by carbon monoxide of steroid-21-hydroxylase from beef adrenal cortex microsomes, a mixed function oxidase enzyme, was released by light, the most effective wavelength for the release of inhibition being 450 nm (12). Many other mixed function oxidase reactions have been shown to involve cytochrome P450, by use of the action spectrum of the release of carbon monoxide inhibition. These include the 11β-hydroxylation

of deoxycorticosterone,[13] and the side-chain cleavage of cholesterol (14) in the bovine adrenal cortex mitochondria, the demethylation of aminopyrine and codeine, and the hydroxylation of acetanilide and testosterone at the 6β, 7α, and 16β positions (15,16) in liver microsomes.

Cytochrome P450 is not the only component of the mixed function oxidase enzyme system. A flavoprotein, reducible with NADPH, is thought to transfer electrons, possibly via an unknown intermediate, to the P450 (17). There is also an absolute requirement for a phospholipid component for the NADPH-cytochrome P450 reduction to occur (18). Recently, cytochrome b_5 which is present in large amounts in the liver microsomes has been implicated in the action of P450-dependent liver microsomal hydroxylases (19), but it may not play an essential role as systems have been prepared which lack cytochrome b_5 and carry out hydroxylation (20).

The cytochrome P450 dependent mixed function oxidase enzymes of liver microsomes are highly inducible. If phenobarbital, for instance, is injected into a rat for several days, the liver microsomal cytochrome P450 level, the NADPH-cytochrome P450 reductase level, the "non-specific" hydroxylase level and the level of many other enzymes will increase by up to five fold, (17). The same is true of the administration of many other drugs which can be hydroxylated, each substrate inducing its own hydroxylation activity and that of many other substrates at the same time. This problem of cross-induction is complicated by the large numbers of compounds which have been tested, by sex, strain, and age differences

and by the nutritional state of the animals used (21,22).

One of the aims of this study was to discover the relationship between cholesterol-7α-hydroxylation and the liver microsomal cytochrome P450. As has been pointed out, both cholesterol-7α-hydroxylase and cytochrome P450 are highly inducible, and the changes in the P450 and its associated hydroxylations on induction of the cholesterol-7α-hydroxylase with cholestyramine feeding were investigated.

One of the most useful methods of investigation of cytochrome P450 is spectrophotometry. Many compounds, when added to a suspension of liver microsomes, produce changes in the oxidised spectrum of P450. Spectral changes can be produced on the addition of substrates of P450 dependent oxidations, the spectral binding constants being closely related to the Michaelis' constants determined for the hydroxylation of these substrates (23). Other compounds which are not substrates of P450 dependent oxidations, notably amines, produce spectral changes (24). The spectral changes induced in the oxidised P450 are of two types commonly known as Types I and II. Using a difference spectrum technique (described in Section 2) a Type I spectrum has absorption maximum at 390 nm and a minimum at 420 nm. A Type II spectrum is the inverse, having a maximum at 420 nm and a minimum at 390 nm. Aminopyrine and hexobarbital, for example, form Type I difference spectra and aniline and several alcohols form Type II difference spectra. The presence of one substance which induces a spectral change will interfere with the production of a spectral change by

another (25). Therefore it is possible to infer the amount of binding of one substrate to the cytochrome P450 by the use of another substrate. This becomes an important technique in the case of cholesterol as the substrate of the P450 dependent oxidation, as all tissues contain large amounts of cholesterol which cannot be easily removed (93). By using other substrates, changes in the state of the P450 on induction of the cholesterol-7α-hydroxylase were investigated.

As is the case with all membrane bound enzymes, the investigation of the properties of cholesterol-7α-hydroxylase is greatly hampered by the particulate nature of the microsomes, and part of the work of this investigation was involved in attempts to release the components of the cholesterol-7α-hydroxylase into a soluble form, with a view to fractionation and purification of the enzyme. Much work has been done on the solubilisation of the cytochrome P450 and the drug hydroxylase activity of the liver microsomes and techniques developed in those and other studies of membrane bound enzymes were applied to the cholesterol-7α-hydroxylase (20,26).

Mitton, during previous work on the 7α-hydroxylation of cholesterol in this laboratory, found that if the liver microsomes are incubated with radioactive tracer cholesterol and NADPH, radioactive products as well as 7α-hydroxy cholesterol are formed (5). These oxidised cholesterol products include cholest-5-en-3β,7β-diol (7β-hydroxycholesterol), cholest-5-en-7-one-3βol (7-ketocholesterol) and cholestan-3β,5α,6β-triol. There is no evidence that these

products are produced *in vivo*, and they are not produced from incubation of the 18,000 g supernatant with NADPH and (4-^{14}C) cholesterol. It is well known that NADPH will stimulate the peroxidation of lipids in the liver microsomes, especially in the presence of ferrous ions and ADP. It is thought therefore that the formation of cholesterol 'autoxidation' products is also due to peroxidation reactions (5). The relationship between cholesterol autoxidation and lipid peroxidation has been investigated. The rat liver supernatant can inhibit the production of autoxidation products from cholesterol, and allow the production of 7α-hydroxycholesterol to proceed without interference from other oxidation reactions. It follows that there must be some 'cofactor' in the 105,000 g supernatant which is necessary for the 7α-hydroxylation of cholesterol to proceed. This anti-autoxidation cofactor has been extensively investigated in this study.

The aims of this research can therefore be stated as follows:-

(i) To investigate the relationship between cholesterol-7α-hydroxylase and other mixed function oxidases in the liver microsomes, with special reference to cytochrome P450.

(ii) To examine the relationship between the autoxidation of cholesterol and the peroxidation of lipids in the liver microsomes.

(iii) To investigate the nature of the cholesterol autoxidation inhibitor present in the supernatant of the liver, and to elucidate its mode of action.

(iv) To solubilise and purify the cholesterol-7α-hydroxylase enzyme and to examine its components.

SECTION 2

EXPERIMENTAL METHODS

A. Experimental Animals

Male rats of the Wistar strain, bred in the animal house of this department were used in most of the experiments. Rats from the Bush Estate, Midlothian were used in addition on some occasions. The rats were usually killed at a weight of 150 - 200 g. The diet used was a soft diet consisting of 70% whole meal flour, 25% skimmed milk powder and 5% yeast extract. In experiments where cholesterol 7α-hydroxylase activity was to be measured the animals were fed the soft diet containing 4% of cholestyramine resin. ('CUEMID', Merck, Sharp and Dohme)

B. Preparation of Liver Subfractions

The rats were anaesthetised with ether, and the livers were rapidly excised and placed in ice-cold 0·154 M KCl. In some experiments, when it was desirable to have as little haemoglobin present as possible (e.g. for spectral studies), the portal vein of the animal was ligated and the liver was washed out with 0·154 M KCl at 37° via the portal vein anterior to the ligation. The liver turned from dark red to yellow when the blood was replaced by the KCl solution.

The excised liver was weighed, and homogenised with a glass/teflon homogeniser driven by a low speed motor in ice-cold 0·154 M KCl (4 vols KCl to 1 gram rat liver).

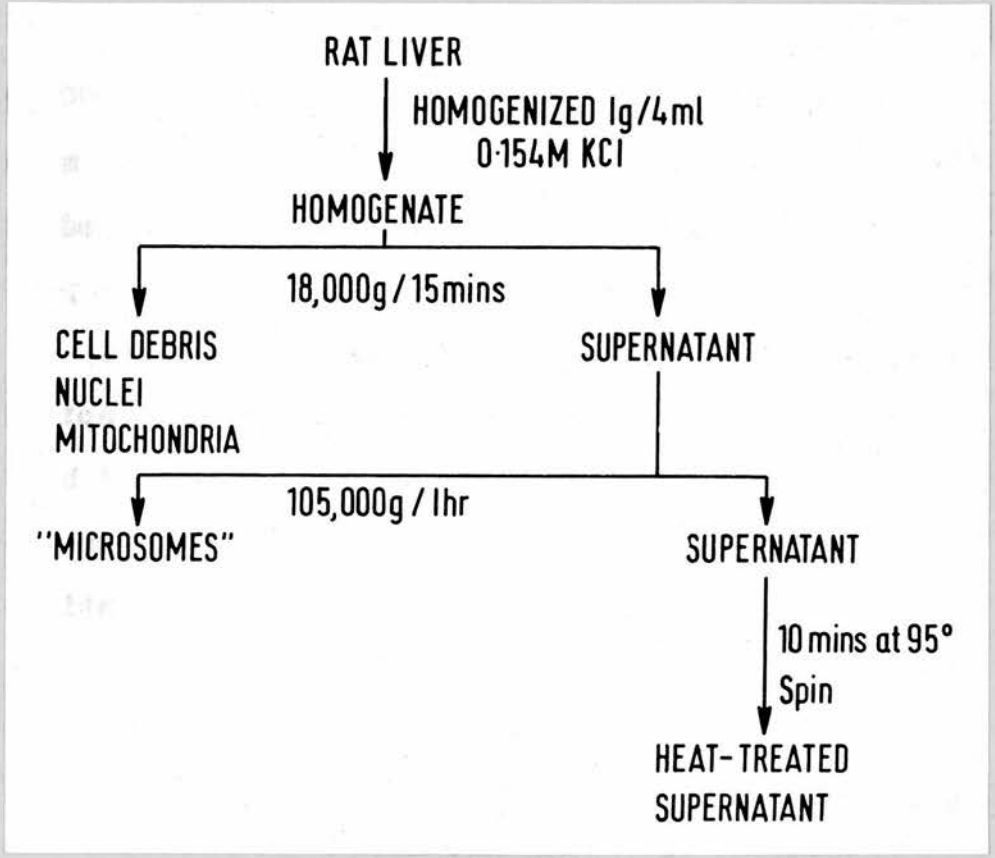

Figure 3. The preparation of liver subfractions.

The centrifugation procedure used to obtain the mitochondrion-free supernatant, the microsomal pellet and the cell sap was as follows.

The liver homogenate was centrifuged at 18,000 g for 15 minutes to precipitate cell debris, nuclei and mitochondria.

The 18,000 g supernatant was then centrifuged at 105,000 g for one hour, sedimenting the microsomes as a red pellet and leaving the clear orange cell sap as supernatant.

The 105,000 g supernatant was heated in a boiling water bath for ten minutes and the denatured protein removed by centrifugation and filtration through glass wool in order to prepare the heat-treated supernatant fraction. The complete fractionation scheme is shown in Figure 3.

C. The Cholesterol Oxidation System

The assay used was a radioactive tracer assay, carried out with resuspended microsomes incubated aerobically with NADPH and (4-^{14}C) cholesterol. The composition of the assay medium was as follows.

1. Microsomal pellet equivalent to 1 g wet weight of tissue homogenised in 1 ml 0·1 M phosphate buffer pH 7·4.
2. NADPH generating system consisting of 5 μmole NADP, 50 μmole Glucose-6-phosphate and one I.U. of Glucose-6-phosphate dehydrogenase in one ml of water.
3. One ml of phosphate buffer 0·1 M pH 7·4 made up from Na_2HPO_4 and NaH_2PO_4.

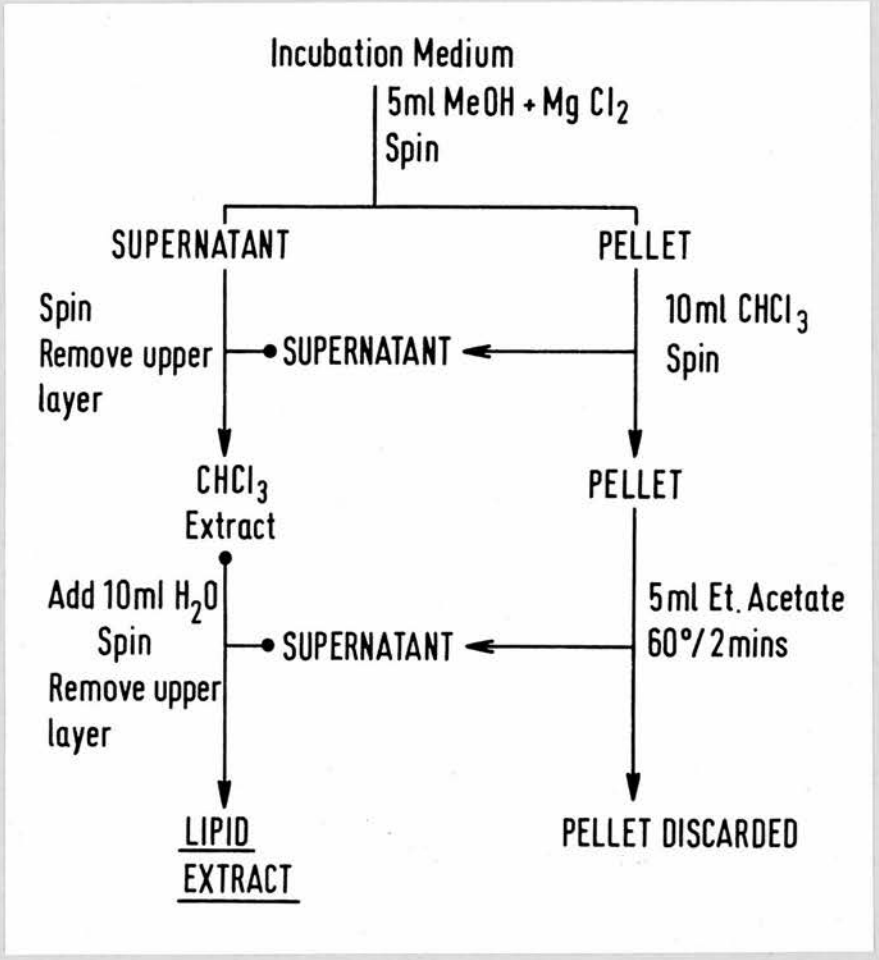

Figure 4. The extraction of radioactive products of (4-^{14}C) cholesterol after incubation with liver microsomes.

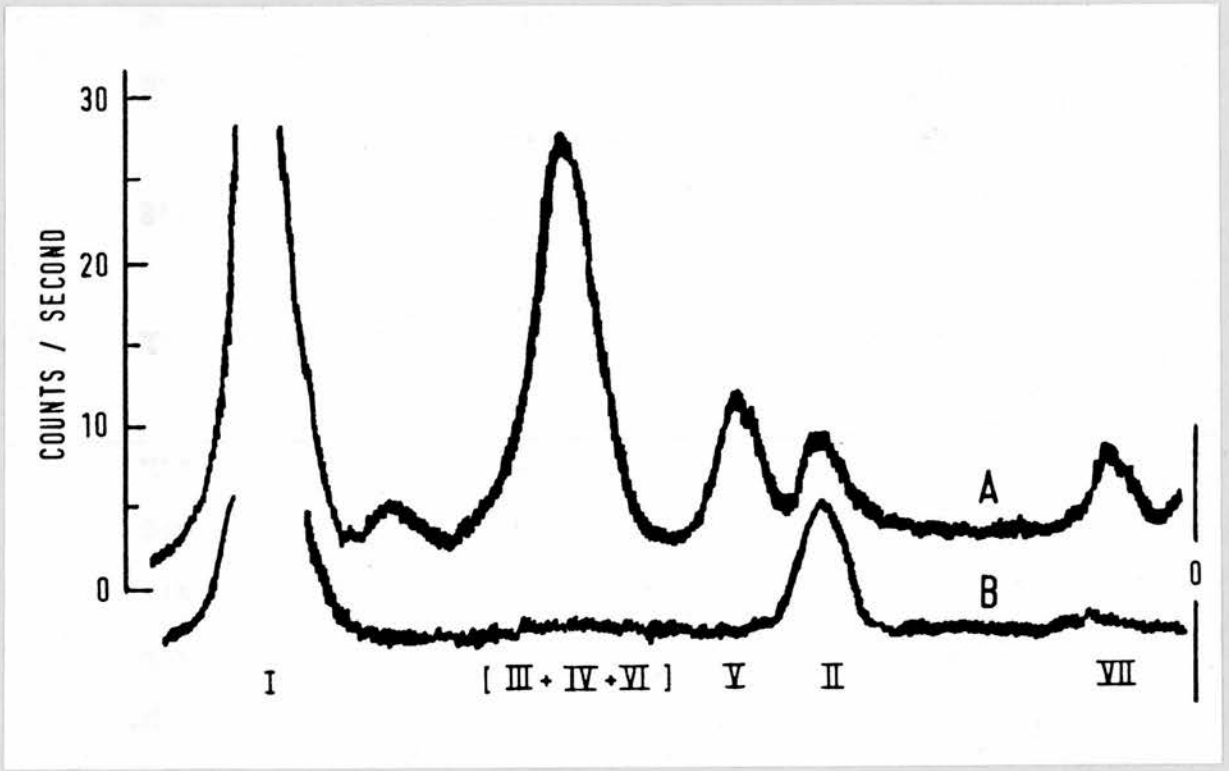

Figure 5. Radioactive scans of thin layer plates after separation of products of incubation of (4-^{14}C) cholesterol with liver microsomes in the absence (A), and the presence (B) of 10 mM-mercapto-ethylamine. The products are identified in Section 5, Figure 20.

4. 4 ml of water or additions made up in aqueous solution.
5. 0·1 μC ($4-^{14}C$) cholesterol in 0·05 ml acetone.

In the case of assay of the cholesterol-7α-hydroxylase activity of the liver microsomes, 10 mM β-mercaptoethylamine was added to the incubation medium to inhibit formation of cholesterol autoxidation products, as described in Section 5.

The mixture was incubated at 37° in a shaking water bath for one hour.

The products of the reaction were measured by organic solvent extraction and Thin Layer Chromatography.

The reaction was stopped by pouring the assay medium into 10 ml methanol containing a crystal of $MgCl_2$ in order to facilitate precipitation. The extraction procedure is shown in Figure 4.

The lipid extract was applied to a thin layer chromatography (TLC) plate of Silica Gel H in a narrow band and the plate was developed in a solvent system of benzene and ethyl acetate in a ratio of 7 to 13.

The radioactive products were located after development using a Panax thin layer scanner, the radioactive peaks being identified by their Rf values. The products obtained were characterised in this laboratory by Mitton, Scholan and Boyd (5). Radioactive scans of the products of the incubation of ($4-^{14}C$) cholesterol with liver microsomes in the presence and absence of 10 mM cysteamine are shown in Figure 5.

Quantitation of the radioactive products was accomplished by removing the radioactive areas of the TLC plate into liquid

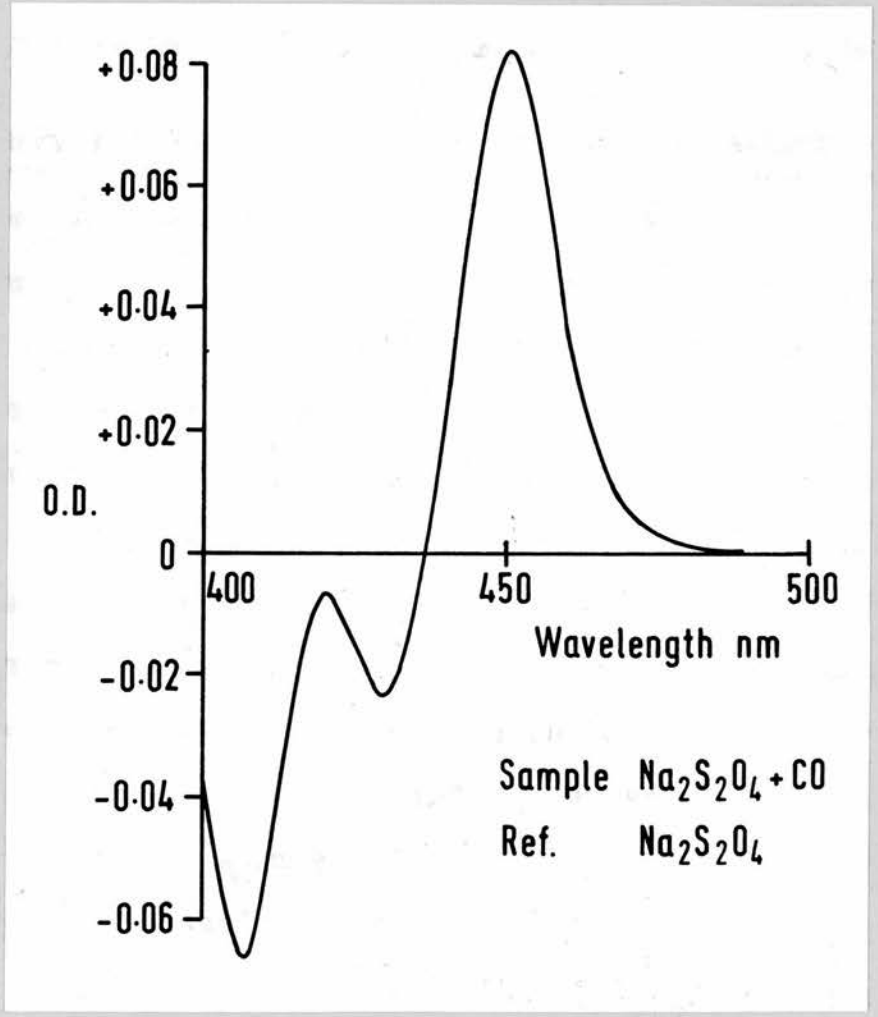

Figure 6. A typical difference spectrum of reduced carbon monoxide treated microsomes against reduced microsomes showing the cytochrome P450 spectrum.

scintillation counting vials and measuring the radioactivity by liquid scintillation in a Packard Tri-Carb Scintillation Spectrometer. Silica gel produces no significant quenching.

The amount of a radioactive product formed was then expressed as a percentage of the total radioactivity recovered.

D. Determination of Cytochrome P450 and Cytochrome b_5.

Cytochrome P450 estimations were carried out by the method of Omura and Sato (11). A suspension of liver microsomes in 0·1 M phosphate buffer pH 7·4 at a concentration of 1 - 2 mg protein per ml was divided into 2 cuvettes which were placed in an SP 800 dual beam spectrophotometer equipped with a scale expander and slave recorder. A scan was made from 500 nm to 390 nm in order to obtain a base line for the P450 difference spectrum. CO gas was then bubbled for 20 - 30 seconds through the sample cuvette, a few grains of sodium dithionite were added to each cuvette, and a second scan was run. The cytochrome P450 concentration was measured by calculating the difference in optical density between 450 nm and 490 nm, allowing for any irregularities in the base line if necessary.

Cytochrome P450 concentration is usually expressed in nmoles per mg protein and this quantity is calculated using a millimolar extinction coefficient of 91 mM^{-1}, and measuring the protein concentration by the Biuret method.

A typical spectrum is shown in Figure 6. The small peak at 420 nm is due to cytochrome P420, a degraded and enzymatically

inactive form of cytochrome P450. The concentration of P450 found in normal rats is 0·2 - 0·4 nmoles per mg protein. The P450 content of the microsomes used in Figure 6 is 0·37 nmoles/mg protein. It should be noted that the cytochrome P450 levels in the microsomes of rats used in this study are lower than those found in studies by other groups of workers, which are of the order of 0·6 to 0·8 nmoles per mg microsomal protein.

The other main cytochrome of the liver microsomes is cytochrome b_5. This cytochrome is measured by its dithionite reduced against air oxidised difference spectrum, which is measured in the same way as the cytochrome P450 difference spectrum. Cytochrome b_5 in the reduced form has an absorption maximum at 424 nm and a minimum at 409 nm. The millimolar extinction coefficient for the OD 424 nm minus the OD 409 is 185 mM^{-1}. The cytochrome b_5 content of the microsomes is expressed in terms of nmoles per milligram microsomal protein. The value for normal rats is 0·4 - 0·6 nmoles/mg protein.

E. Assay of NADPH Oxidase Activity

NADPH oxidase activity is assayed by the measurement of the rate of reduction of a suitable electron acceptor by NADPH, the reaction being catalysed by an NADPH oxidase enzyme. The electron acceptors used were cytochrome c, which absorbs at 550 nm in the reduced state, and dichlorophenol-indophenol (DCPIP) which absorbs at 590 nm in the oxidised form and is bleached in reduction. The rate of the reaction is followed in a Unicam SP 800

recording spectrophotometer in the fixed wavelength mode, set at the appropriate wavelength. Two cuvettes were made up with the following mixture

2·5 ml 0·1 M sodium phosphate buffer pH 7·4

0·2 ml NADPH generator-(1 μmole NADP, 10 μmole G-6-P 0·2 I.U. G-6-P dehydrogenase)

0·2 ml DCPIP (0·3 mg/ml) or cytochrome c (20 mg/ml)

A base line was drawn with a cuvette in the sample and reference positions. The preparation to be tested for enzymic activity was added in a small volume of buffer to the sample cuvette and the mixture rapidly stirred. The rate of the reaction was calculated by the initial slope of the line obtained, which usually remained linear for at least one minute. The rate is expressed in terms of change in optical density per minute per mg protein.

F. CO Inhibition and Light Reversibility of CO Inhibition

Gas mixtures of varying $CO:O_2:N_2$ ratios were made up in gas burettes. The usual incubation mixture was sealed in a flask with a serum cap and the relevant gas mixture was passed into the flask through a hypodermic needle for 10 minutes prior to addition of the radioactive cholesterol, after which the needle was withdrawn.

The light reversibility of the CO inhibition was tested using a cylindrical glass cell mounted in a water jacket and equipped with a magnetic stirrer. This was mounted on an optical bench. Light from a high-pressure Xenon lamp of 500W was focussed on the cell by means of a biconvex lens. Grubb-Parsons interference

filters of various wavelengths were interposed between the light source and the reaction cell. A $CO/N_2/O_2$ atmosphere was obtained as described above. In these experiments, the stable liver microsome acetone powder was used, as the incubations must be done sequentially.

G. Cytochrome P450 Difference Spectra

The Aminco-Chance Spectrophotometer was used for the measurement of difference spectra. Microsomes were suspended in 0·1 M KCl at a concentration of 4 - 5 mg protein per ml. The suspension was divided into two cuvettes and the difference spectrum was obtained by addition of a solution of the compound under study to the sample cuvette and an equivalent volume of solvent to the reference cuvette. The difference spectrum was obtained by scanning the samples at the relevant wavelengths usually from 370 nm to 470 nm.

In order to measure binding constants for substances which produce difference spectra, the spectrophotometer was used in the dual wavelength mode set at the minimum and the maximum of the spectrum in order to obtain the largest changes in optical density.

H. Measurement of Oxygen Uptake by the Liver Microsomes

Oxygen uptake was measured with a Clark oxygen electrode. The volume of the chamber was 3·5 ml and therefore the assay mixture used was exactly the same as that described in Section C, except that all the volumes were halved and the radioactive cholesterol was omitted. The oxygen uptake of the mixture was

followed on a Servoscribe recorder.

The assay chamber was water-jacketed at 37° and all additions were added in as small a volume as possible.

Oxygen uptake was expressed in terms of arbitrary units per minute. As all experiments involving the use of the oxygen electrode were of a comparative nature, the absolute amounts of oxygen used were not required. The oxygen electrode response was not therefore calibrated.

I. Malonaldehyde Production assay

Malonaldehyde is produced by the liver microsomes in the presence of NADPH and O_2 by the peroxidation of unsaturated fatty acids as described by Hochstein and Ernster (28). The assay of malonaldehyde can therefore be used to measure the extent of lipoperoxidation. The method used was the thiobarbituric acid method of Wilbur et al. (29).

The assay mixture was as described in Section C., except that half volumes were used and that the radioactive cholesterol was omitted. After 20 minutes aerobic incubation at 37°, 0·5 ml of the incubation medium was pipetted into a centrifuge tube containing 2 ml of 30% trichloroacetic acid and 0·2 ml of 5 N hydrochloric acid, and well mixed. 2 ml of 0·75% thiobarbituric acid solution was then added and the mixture was heated in a boiling water bath for 20 minutes. The tube was then centrifuged and the supernatant was decanted into an optical cuvette and read at 535 nm in a Unicam SP 600 spectrophotometer. Using an extinction coefficient

of 1·56 mM^{-1} the malonaldehyde production per milligram of protein per minute was calculated.

J. Assay of aminopyrine demethylase activity

Aminopyrine is hydroxylated in the liver microsomes; an N-methyl group is hydroxylated and further oxidation leads to the demethylation of the nitrogen atom with simultaneous release of formaldehyde (30). The formaldehyde released was assayed by the method of Nash (31). The assay mixture used was the same as that described in Section C, except that aminopyrine was added as substrate and the radioactive cholesterol was omitted. After a 20 minute incubation at 37°, 0·5 ml of 60% TCA was added and the denatured protein was removed by centrifugation. 7 ml of the formaldehyde reagent (2M ammonium acetate, 0·05 M acetic acid and 0·02 M acetylacetone) was added to the supernatant and the mixture was incubated for a further 20 minutes at 37°. The optical density of the mixture at 412 nm was then read in a Unicam SP 600 spectrophotometer. Using an extinction coefficient of 8×10^3 M^{-1}, the production of formaldehyde per minute per milligram of protein was calculated.

K. Assay of Aniline Hydroxylase Activity

Aniline is hydroxylated to p-aminophenol, which was assayed by the method of Brodie and Axelrod (32), as modified by Kato and Gillette (33). The assay mixture was as described in Section C, except that aniline was used as the substrate. After a 30 minute

incubation, the mixture was shaken with 5 ml ether and 1 g NaCl, and centrifuged. 4 ml of the ether layer was aspirated into 3 ml of 0·1 M NaOH containing 1% phenol. The mixture was shaken, and after 30 minutes the alkaline layer was decanted and the optical density read at 620 nm in an SP 600 spectrophotometer. The colour formed was phenol indo-phenol. The activity is expressed in terms of OD 620/min/mg protein.

L. Estimation of Thiol Concentration

The reagent used for the estimation of thiol groups was Ellman's reagent (5,5' dithio-bis(2-nitrobenzoic acid)) 2·4 ml of a 1 mM solution of the reagent in 0·1 M phosphate buffer pH 7·4 was used. To this was added up to 0·1 ml of the solution under test. The yellow colour obtained was measured at 412 nm in a Unicam SP 600 spectrophotometer. The reagent was calibrated using standard solutions of reduced glutathione and β-mercaptoethylamine. These standard curves gave an extinction coefficient very close to the literature value of 13·4 mM^{-1} (97).

M. Sephadex Chromatography

The materials used were Sephadex G 200, G 25, and DEAE Sephadex A 25 from Pharmacia. These were all swollen in 0·1 M phosphate buffer pH 7·4 for at least 2 days before use. The columns used were of two sizes, 2·5 x 30 cm and 5 x 30 cm. The effluent of the column in use was monitored at 278 nm by a Uvicord recording spectrophotometer, and the fractions were

collected in tubes by an automatic Ultrorac fraction collector.

The sample to be fractionated was added in a small volume, and washed on with the column eluant. The columns were run in a downwards direction at atmospheric pressure in a cold room at 4 - 6°.

In the case of the DEAE Sephadex A 25 chromatography, the eluant of 0·02 M phosphate buffer pH 7·0 contained a linear gradient of 0 - 0·5 M KCl, from a gradient mixer.

N. Preparation of Acetone Powder

An acetone powder of rat liver microsomes was used in some experiments and was prepared as follows. The liver microsomes, after sedimentation by centrifugation as described in Section B, were resuspended in a small volume of 0·154 M KCl and decanted rapidly into 15 - 20 volumes of acetone at -20° with vigorous stirring. The mixture was then left at -20° for 10 minutes. The powder was then filtered off in a Buchner funnel and washed twice with acetone and twice with ether all at -20°. The remaining ether was then removed under vacuum in a vacuum desiccator. The powder formed was then stored at -20°, and retained its activity for some weeks.

MATERIALS

All the chemicals used were of the Analar grade and were supplied by BDH or Sigma. Glucose-6-phosphate dehydrogenase was supplied by Boehringer. The toluene used for liquid scintillation counting was washed with concentrated sulphuric acid, and then with water, and dried over sodium sulphate before use. Acetone was distilled after 3 - 4 hours refluxing with potassium permanganate.

SECTION 3

CHOLESTEROL-7α-HYDROXYLASE AS A LIVER MICROSOMAL MIXED-FUNCTION OXIDASE.

A. Introduction

Many lipid and lipid-soluble compounds are hydroxylated in the liver microsomes (10), and cholesterol is no exception in this respect (5). Many of the hydroxylase activities found in the liver microsomes are of the type called 'mixed-function oxidases'. These oxidases require the presence of reducing equivalents, usually from NADPH, and molecular oxygen. The general form of the reaction catalysed by mixed function oxidases is as follows:-

$$RH + NADPH + O_2 + H^+ \longrightarrow ROH + NADP^+ + H_2O \quad (8)$$

The mixed function oxidases of the liver microsomes are in many cases known to have as one of their components cytochrome P450, one of the principal cytochromes of the liver microsomes. This cytochrome is the site of the interaction of oxygen and the molecule undergoing hydroxylation. This cytochrome in its reduced state has an affinity for carbon monoxide, the reduced CO chromophore having a strongly absorbing Soret band at 450 nm. This CO binding property is used to demonstrate the P450 dependence of a mixed function oxidase, the hydroxylation being inhibited by CO and the inhibition being reversed by light at 450 nm if the enzyme contains P450 as an active component. The P450 dependence of the 7α-hydroxylase of cholesterol is described in this section.

At present, it is envisaged that P450-dependent mixed-function oxidases consist of at least two components, which form an electron

transport chain. The first component is a flavoprotein, which has NADPH oxidase activity when supplied with a suitable electron acceptor. The natural electron acceptor is cytochrome P450, but other compounds of appropriate redox potential can accept electrons from NADPH via the flavoprotein. Examples of these are cytochrome c and dichlorophenol indophenol. The flavoprotein was, in fact, discovered as an NADPH-cytochrome c reductase (34). It follows that if either of these non-physiological electron acceptors is incorporated in the incubation mixture in excess, they will compete with the P450 for electrons and effectively inhibit the mixed function oxidation reaction, by 'draining' electrons away from the P450 (40). The effect of cytochrome c on cholesterol-7α-hydroxylase is demonstrated.

It has already been shown in this laboratory that the P450 content of the liver microsomes does not increase on induction of the cholesterol-7α-hydroxylase by cholestyramine feeding or bile duct cannulation (35), nor does the hydroxylase activity increase on induction of cytochrome P450 by phenobarbitone treatment, which increases many drug hydroxylating activities. There are several possible explanations of this result. It is possible that, although the total cytochrome P450 content of the liver microsomes does not change, there is a change in the relative amounts of two or more subspecies of the cytochrome. Several groups of workers, by various techniques, have shown the presence of a plurality of types (36,37). Another possibility is that the hydroxylation of cholesterol is carried out by a subspecies of cytochrome P450.

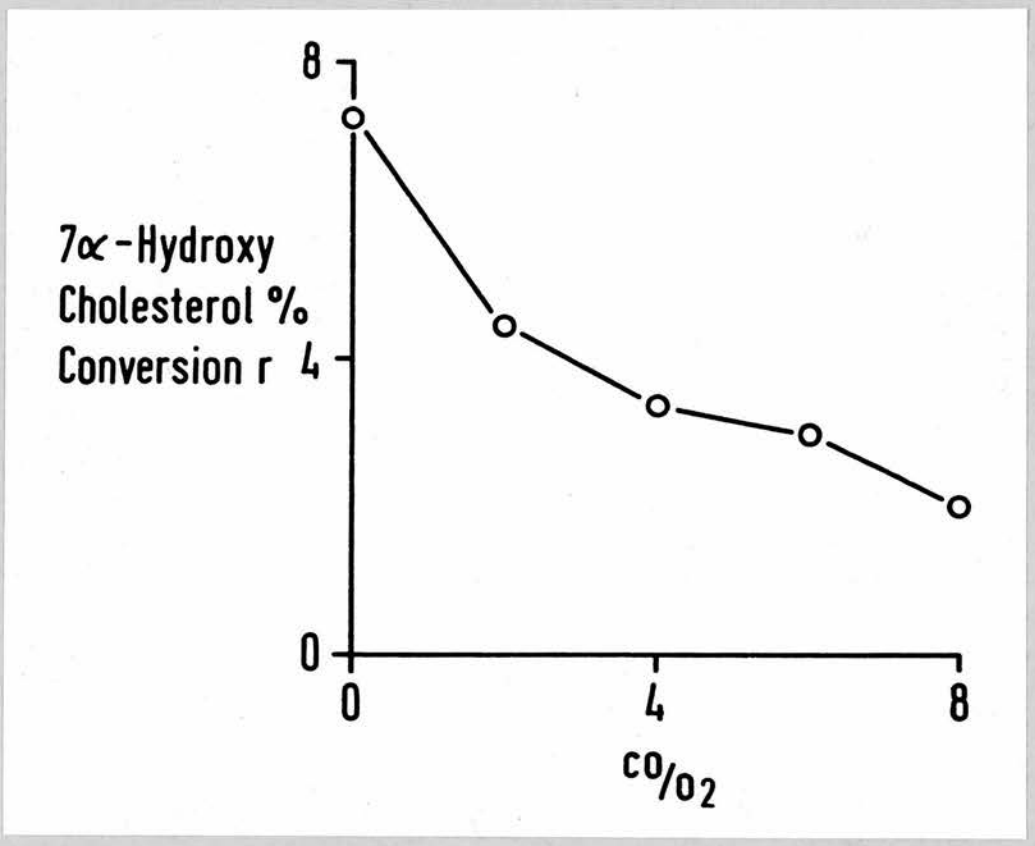

Figure 7. The inhibition of cholesterol-7α-hydroxylase in liver microsome acetone powder by carbon monoxide.

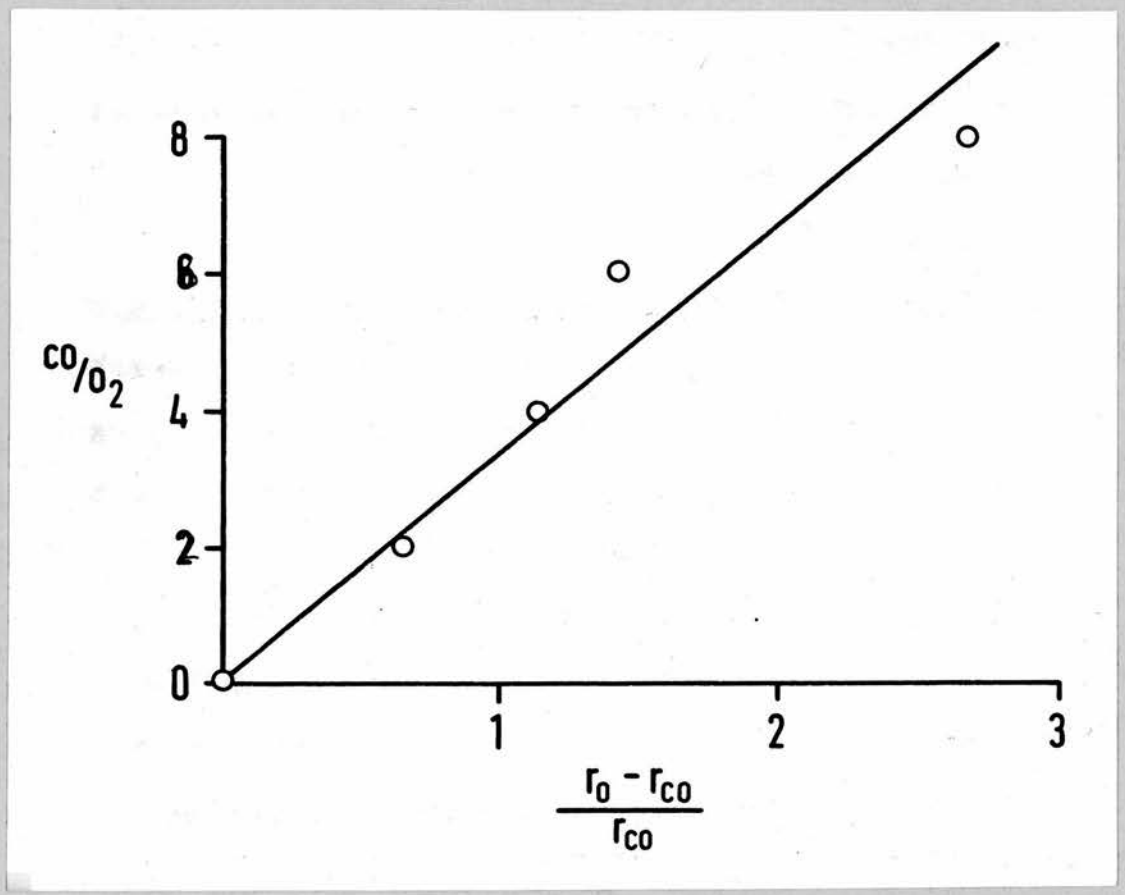

Figure 8. The calculation of the Warburg partition constant for the cholesterol-7α-hydroxylase.

In this case a rise in this sub-species could be too small to measure in the presence of the large amounts of non-specific, cytochrome P450. Both these possibilities were tested to some extent by investigation of the spectral and metabolic activities of the microsomes.

B. The Carbon Monoxide Inhibition of the Cholesterol-7α-Hydroxylase

Otto Warburg, in his book 'Heavy Metal Prosthetic Groups' (38), formulated the theory of oxygen interaction with haem-containing enzymes and the inhibition of these enzymes by carbon monoxide, thus putting into mathematical terms the observation of Bernard, in the nineteenth century, that carbon monoxide competes with oxygen in biological systems (39). In the case of cholesterol-7α-hydroxylase, the enzyme was shown to be inhibited by CO, as shown in Figure 7.

If it is assumed that the carbon monoxide is competing with oxygen for a site on the enzyme, then the following relationship holds

$$E.O_2 + CO \rightleftharpoons E.CO + O_2$$

Therefore

$$K = \frac{(E.O_2)(CO)}{(E.CO)\ (O_2)}$$

K is known as the Warburg Partition Constant, and it can be calculated from the data shown in Figure 7 by plotting CO/O_2 against $\frac{r_o - r_{co}}{r_{co}}$, where r_o is the reaction rate in the absence of CO and r_{co} is the rate in its presence. The result of such a plot is shown in Figure 8, and from the graph it can be calculated that

the partition constant is 3·3 for this particular microsome acetone powder preparation. It was found that the partition constant varied from preparation to preparation, the value always lying between 2 and 5. A similar variation has also been found by other workers using different enzyme systems (14,16).

The result obtained is however of the same order of magnitude as that found for several P450 dependent mixed function oxidases, and is in contrast to partition constants of about 6×10^{-3} for haemoglobin and 10 for cytochrome oxidase.

C. The Light Reversibility of Carbon Monoxide Inhibition

In his book, Warburg also pointed out that carbon monoxide-haem complexes could be dissociated by light, thus relieving the inhibition caused by CO. Estabrook et al. showed that the CO inhibition of steroid 21-hydroxylase can be released by light, the most effective wavelength being 450 nm. This is taken as strong evidence for the involvement of cytochrome P450 in the enzyme (12).

The results shown in Table 1 show that the carbon monoxide inhibition of cholesterol-7α-hydroxylase is reversed by white light, and that furthermore the most effective wavelength for reversal of the inhibition is near 450 nm.

The results are best presented as a photochemical action spectrum which is calculated as follows.

Table 1

The effect of light of various wavelengths on the carbon monoxide inhibition of cholesterol-7α-hydroxylase.

Atmosphere	Light Wavelength nm	Percentage conversion to 7α-hydroxycholesterol
Air	No light	10·83
$CO:O_2:N_2$::50:10:40	No light	3·16
"	410	3·86
"	429	4·13
"	448	5·71
"	470	4·06
"	490	3·44

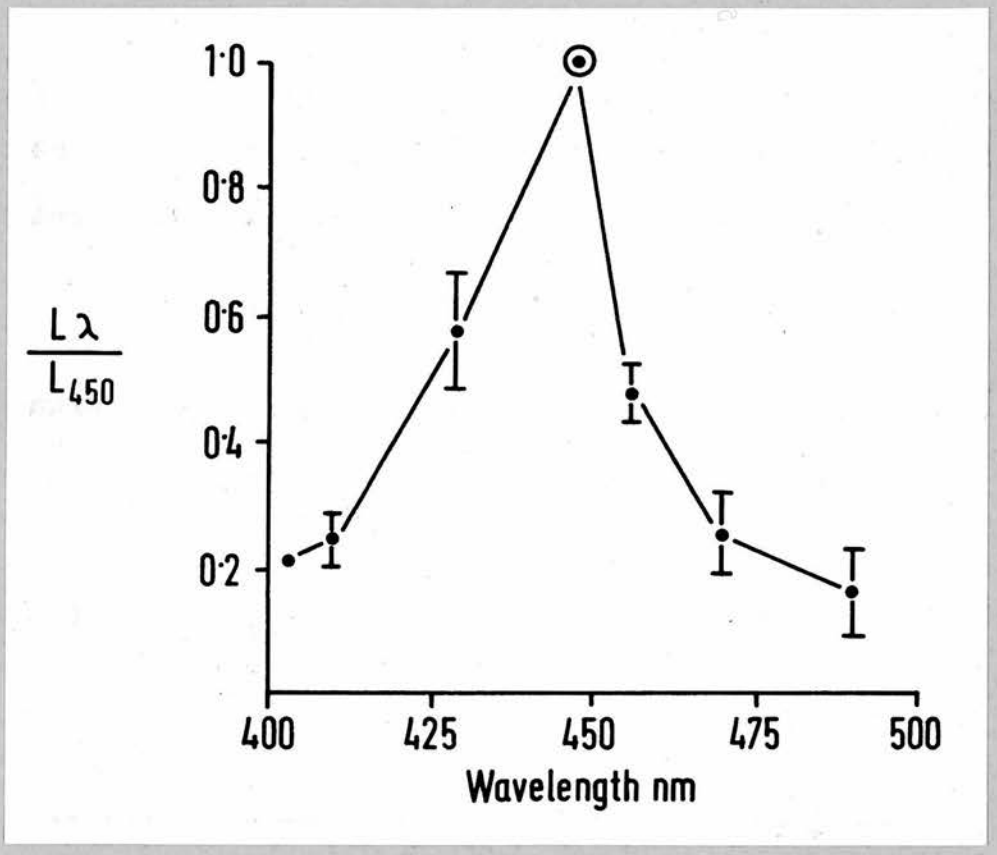

Figure 9. The photochemical action spectrum of cholesterol-7α-hydroxylase in rat liver microsome acetone powder.

The Warburg partition constant in the dark K_d is given by:-

$$K_d = \left(\frac{r_{co}}{r_o - r_{co}}\right)_d \cdot \frac{CO}{O_2}$$

where subscript d refers to reactions in the absence of light.

On exposure to light, a new constant is obtained:-

$$K_l = \left(\frac{r_{co}}{r_o - r_{co}}\right)_l \cdot \frac{CO}{O_2}$$

Therefore the partial change in the partition constant

$$\frac{\Delta K}{K_d} = \frac{K_l - K_d}{K_d}$$

This is proportional to the light intensity i for any wavelength, therefore

$$\frac{\Delta K}{K_d} = L.i$$

L is the light sensitivity. The photochemical action spectrum is plotted therefore in terms of L_λ / L_{450} against wavelength.

The spectrum obtained from many experiments is shown in Figure 9 and shows a marked peak at near 450 nm, all other wavelengths tested being relatively ineffective.

This provides strong evidence that the carbon monoxide chromophore which absorbs light at 450 nm is an active component in the cholesterol 7α-hydroxylase.

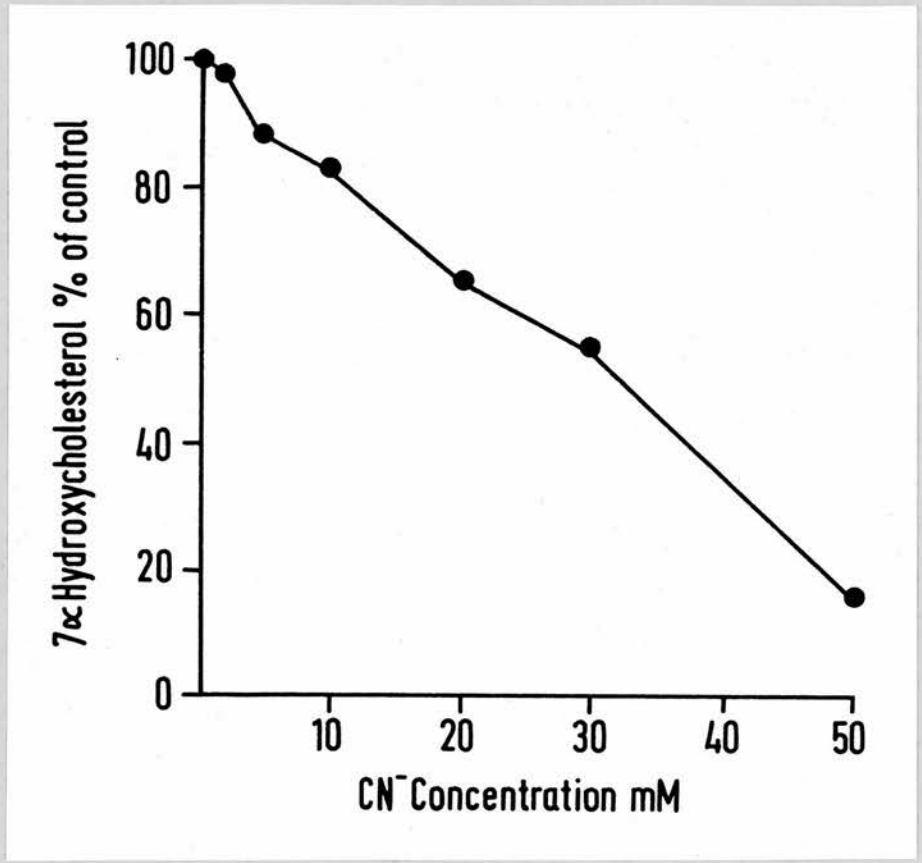

Figure 10. The effect of cyanide ions on the cholesterol-7α-hydroxylase of rat liver microsomes.

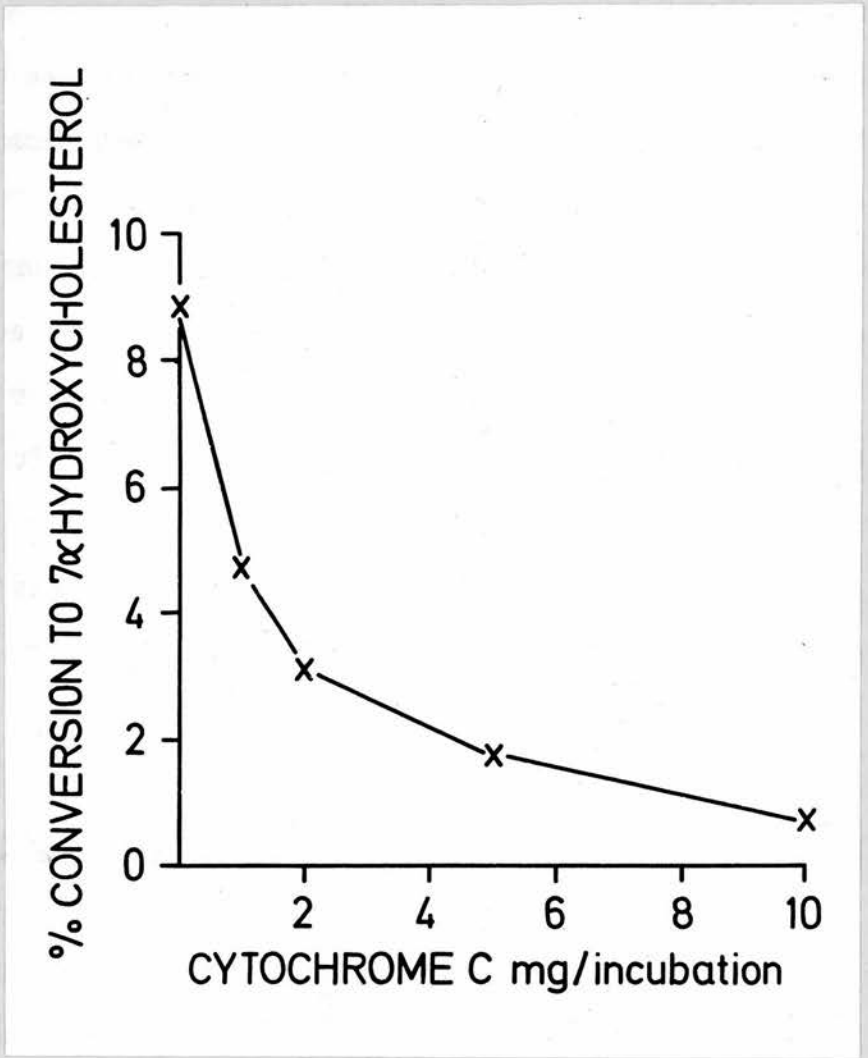

Figure 11. The effect of cytochrome c on the cholesterol-7α-hydroxylase of rat liver microsomes.

D. The Effect of Cyanide Ions on the Cholesterol-7α-Hydroxylase Enzyme.

A further piece of evidence that cytochrome P450 is involved in the cholesterol-7α-hydroxylase enzyme system comes from the observation of the effect of cyanide ions on the enzyme system. Several enzymes which are inhibited by carbon monoxide are also inhibited by cyanide, e.g. cytochrome oxidase. The effect of cyanide on the cholesterol-7α-hydroxylase is shown in Figure 10. The inhibition becomes significant at very high concentrations of cyanide only, 50% inhibition occurring at greater than 30 mM. This is in common with other liver microsomal mixed function oxidases which are dependent on cytochrome P450 for their action (40) and in contrast to the liver microsomal cyanide sensitive oxidative enzymes such as lanosterol demethylase, which do not involve cytochrome P450 (41).

E. The Effect of Cytochrome c on Cholesterol-7α-Hydroxylase

Cytochrome c is used as an inhibitor of reactions which involve the NADPH-cytochrome c reductase flavoprotein (40). This flavoprotein's physiological function is to act as an NADPH-cytochrome P450 reductase. The effect of various concentrations of cytochrome c on the cholesterol-7α-hydroxylase enzyme is shown in Figure 11. It can be seen that there is marked inhibition of the activity of the enzyme, thus indicating that the reducing equivalents from NADPH are being removed from the system and therefore not partaking in the hydroxylation reaction. This gives an

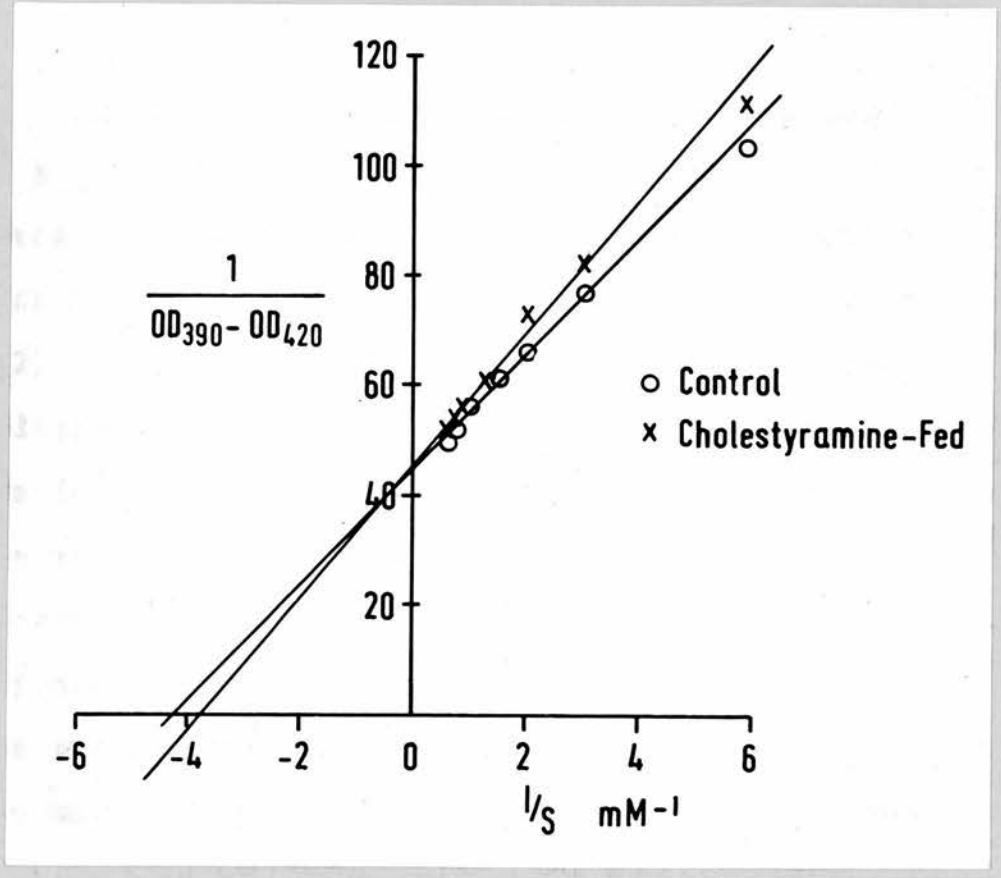

Figure 12. Lineweaver-Burke plots of aminopyrine concentration against the optical density change between 390 nm and 420 nm in liver microsomes from control and cholestyramine-fed rats.

indication that the NADPH-cytochrome c reductase flavoprotein is involved in the cholesterol-7α-hydroxylase enzyme complex.

F. Aminopyrine Difference Spectra and Metabolism

Aminopyrine is oxidatively demethylated by a cytochrome P450 mixed function oxidase enzyme in the liver microsomes (15). Addition of aminopyrine to the microsomes causes a Type I shift in the oxidised P450 spectrum. The Michaelis constant (K_m) and the maximum velocity (V_{max}) of the demethylation reaction were determined by measuring the velocity of production of formaldehyde at several different aminopyrine concentrations. Lineweaver-Burke plots of reciprocal substrate concentration against reciprocal initial velocity were drawn, and the K_m and V_{max} determined in the usual way. The determination of spectral dissociation constant (K_s) for aminopyrine was carried out in a similar manner, the reciprocal of the optical density difference between 390 nm and 420 nm being plotted against the reciprocal substrate concentration. These determinations were carried out on control and cholestyramine-fed rats, five rats in each group. An example of the determination of the K_s of aminopyrine in microsomes from one control and one cholestyramine-fed rat is shown in Figure 12. Table 2 gives the full results. As can be seen there are no significant differences in any of the parameters between the control and cholestyramine-fed groups.

Table 2

Metabolic and spectral parameters for aminopyrine in liver microsomes from control and cholestyramine-fed rats.

		Control	Cholestyramine-fed
K_s	mM	0·23 ± 0·03	0·22 ± 0·03
K_m	mM	1·77 ± 0·41	2·08 ± 0·29
V_{max}	nmoles/min/mg protein	1·46 ± 0·40	1·62 ± 0·30

Table 3

Metabolic and spectral parameters for aniline in liver microsomes from control and cholestyramine-fed rats.

		Control	Cholestyramine-fed
K_s	mM	0·42 ± 0·11	0·32 ± 0·03
K_m	mM	0·52 ± 0·13	0·75 ± 0·23
V_{max}	OD_{620}/min/mg protein	0·097 ± 0·028	0·090 ± 0·010

G. Aniline Difference Spectra and Metabolism

Aniline is hydroxylated by a P450 dependent mixed function oxidase in the liver microsomes to p-aminophenol. It causes a Type II shift in the oxidised P450 spectrum. Determinations of K_m and V_{max} were carried out for aniline hydroxylation in the same way as those for aminopyrine demethylation. p-Aminophenol is assayed by measuring the optical density at 620 nm of a phenol p-aminophenol complex. The K_s is determined in the same way as that for aminopyrine. The determinations were carried out on liver microsomes from control and cholestyramine-fed rats four animals in each group. The results are given in Table 3. Once again there is no significant difference between the control and cholesterol-7α-hydroxylase induced animals.

H. n-Octylamine Difference Spectra

Jefcoate and Gaylor (36) showed that n-octylamine, in common with many other amines forms a Type II difference spectrum with the liver microsomal P450. However, the difference spectrum formed by n-octylamine has two minimum values, at 392 nm and 410 nm. The ratio of these minima varies according to the pretreatment of the experimental animals, in a way which indicates that each of the minima is related to a separate type of cytochrome P450. For example, in microsomes from phenobarbitone-treated rats, the 410 nm minimum predominates, and in microsomes from rats pretreated with methylcholanthrene, the 392 nm minimum predominates. These two types of n-octylamine binding have been correlated to the two spin

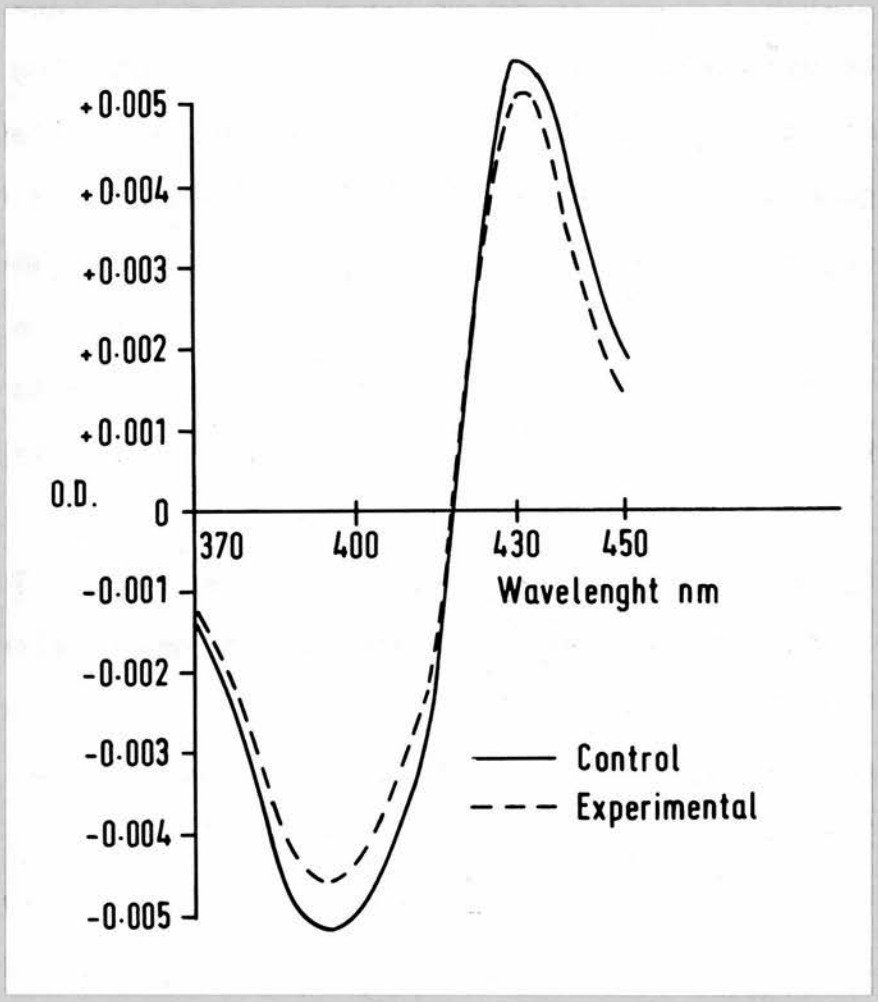

Figure 13. n-Octylamine difference spectra of microsomes from control and cholestyramine-fed rats, at 1 mM n-octylamine.

states of the ferric iron atom in the haem of the cytochrome P450, described by Hildebrandt et al.(42) as high- and low-spin. Using the relative absorbancies at 392 nm and 410 nm of the n-octylamine difference spectrum the ratio of high to low spin P450 can be calculated. This ratio is found to be 1:3 high- to low-spin in normal microsomes and 1:1 in microsomes from rats treated with methylcholanthrene (36). The n-octylamine difference spectrum of microsomes from rats fed on cholestyramine, at a concentration of 1 mM n-octylamine, showed no significant difference in the OD_{392} to OD_{410} ratio from that of normal rats, as shown in Figure 13, thus demonstrating that the ratio of high- to low-spin P450 is not altered on induction of the cholesterol-7α-hydroxylase enzyme.

I. Effect of P450 Substrates on the Cholesterol-7α-Hydroxylase

Many of the compounds known to interact with cytochrome P450 will inhibit the hydroxylation or spectrum formation of other P450 substrates (25). This is probably due to competition for the P450 binding site. Various compounds which are hydroxylated in the liver were tested for their effect on the cholesterol-7α-hydroxylase to discover if there is any inhibition of the activity. If inhibition is to occur by competition for the P450 binding site, then it would be expected to occur at concentrations approaching the K_m for hydroxylation or K_s for spectral binding of the substance under test. It is possible however that inhibition could occur at higher concentrations, by competition for reducing equivalents from NADPH. Aniline and aminopyrine were tested at various

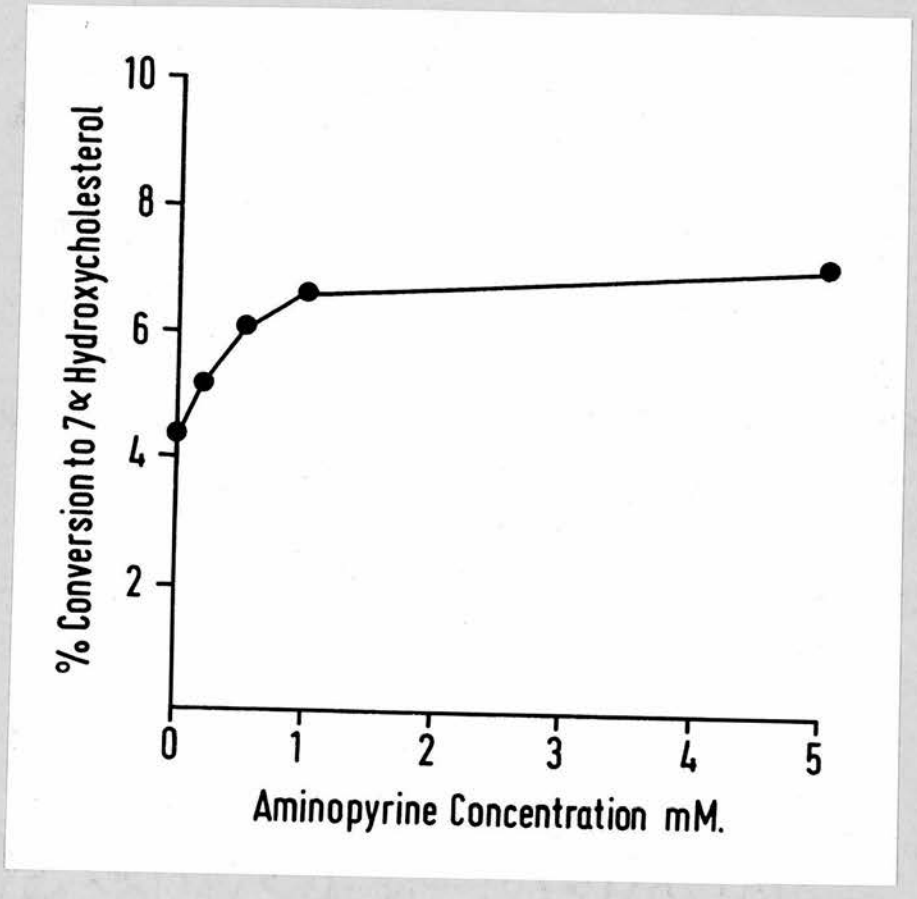

Figure 14. The effect of aminopyrine on the cholesterol-7α-hydroxylase of rat liver microsomes.

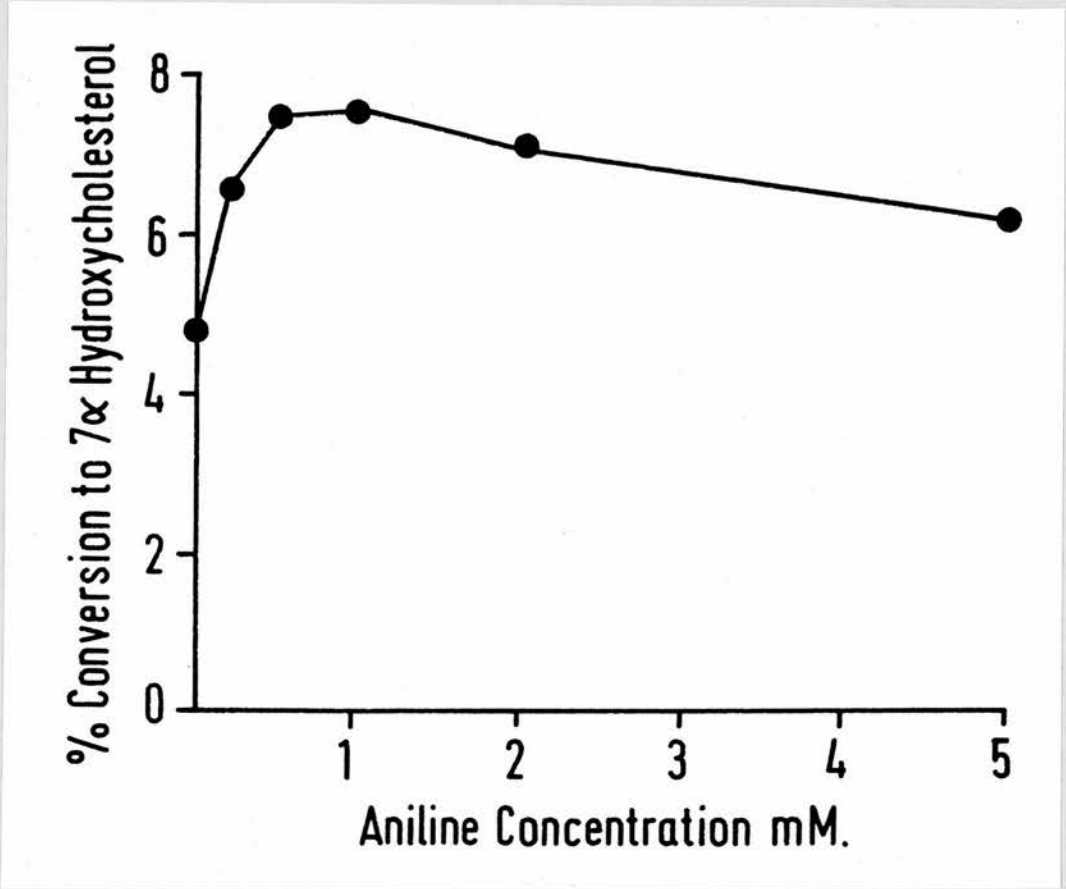

Figure 15. The effect of aniline on the cholesterol-7α-hydroxylase of rat liver microsomes.

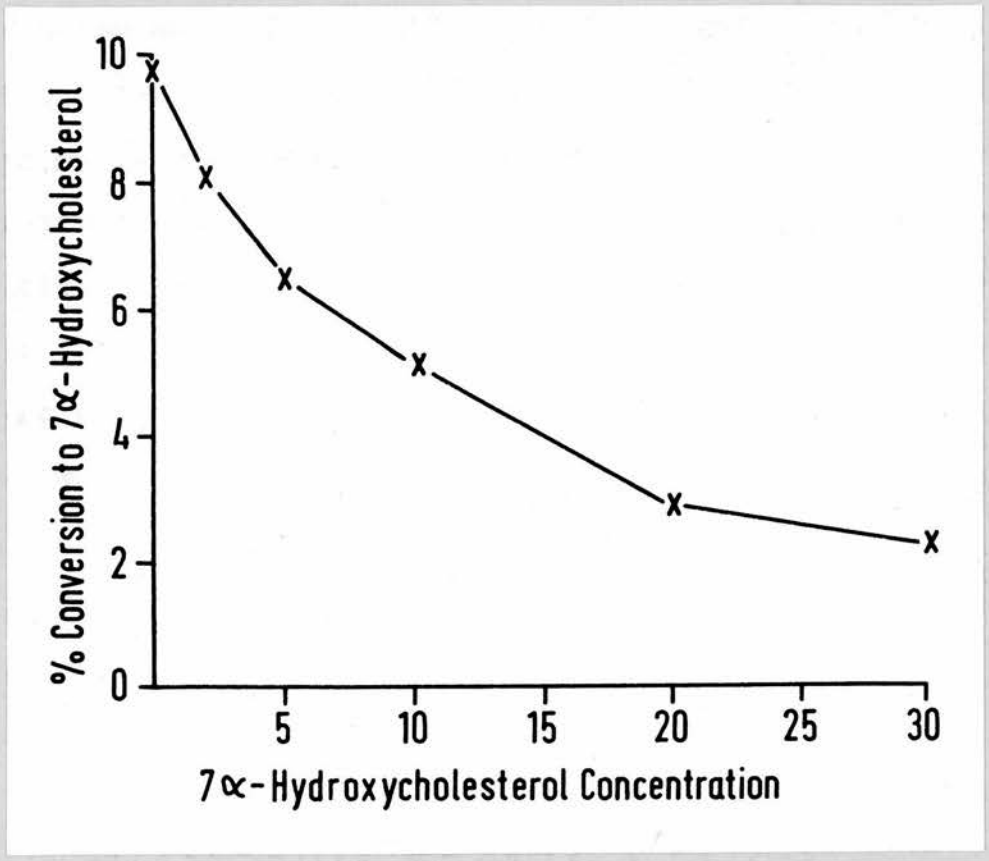

Figure 16. The effect of 7α-hydroxycholesterol on the cholesterol-7α-hydroxylase of rat liver microsomes.

concentrations against the cholesterol-7α-hydroxylase, and the results are shown in Figures 14 and 15. An unexpected result is obtained in that the cholesterol-7α-hydroxylase activity is stimulated in the presence of the compounds which are undergoing hydroxylation concomitantly. The reason for this result is obscure, but it shows, at least, that there is no competition for the same cytochrome P450 binding site. It is interesting to note that β-mercaptoethylamine, which is used routinely in the assay of cholesterol-7α-hydroxylase activity has been shown to have a spectral binding constant with P450 of 1 mM (36). At 10 mM, the concentration used in the incubation mixture, the binding site will be completely saturated, yet the cholesterol-7α-hydroxylase activity is at a maximum.

J. Effect of 7α-Hydroxycholesterol on the Cholesterol-7α-Hydroxylase

In the adrenal cortex mitochondria, it is found that pregnenolone, the product of the P450 dependent side-chain cleavage reaction of cholesterol, produces a strong Type I difference spectrum when added to the mitochondria (94). 7α-Hydroxycholesterol, when added to the liver microsomes from control or cholestyramine-fed rats, produces no difference spectrum up to concentrations saturating the aqueous phase. 7α-Hydroxycholesterol will however cause a marked inhibition of the cholesterol-7α-hydroxylase as is shown in Figure 16. It is impossible to investigate whether the type of inhibition caused by 7α-hydroxycholesterol is competitive

or non-competitive because of the presence of saturating concentrations of cholesterol at all times in the incubation medium.

SUMMARY OF RESULTS

1) The cholesterol-7α-hydroxylase enzyme is inhibited by carbon monoxide.

2) The inhibition by carbon monoxide is reversed by light at 450 nm leading to the conclusion that the enzyme is dependent on cytochrome P450 for its activity.

3) The enzyme is not strongly inhibited by cyanide.

4) The enzyme is inhibited by cytochrome c.

5) The metabolism and spectral binding properties of aminopyrine and aniline are the same in normal and cholesterol-7α-hydroxylase induced rats.

6) The ratio of high-spin to low-spin P450 in the control and induced rats, as measured by the n-octylamine method, is the same.

7) Aminopyrine and aniline stimulate the production of 7α-hydroxy cholesterol.

8) 7α-Hydroxycholesterol produces no spectral change in the liver microsomes, but it causes a marked inhibition of the cholesterol-7α-hydroxylase.

SECTION 4

CHARACTERISATION OF THE CHOLESTEROL-7α-HYDROXYLASE OF RAT LIVER MICROSOMES

A. Introduction

In Section 3 the cholesterol-7α-hydroxylase enzyme was shown to be a cytochrome P450 dependent mixed function oxidase of the liver microsomes. Since the discovery of the P450 dependent mixed function oxidases, great interest has been shown in the problem of obtaining a soluble preparation of these enzymes, with a view to elucidating their properties and to fractionating the multicomponent system into its constituent parts. One of the major difficulties in this task is the extreme lability of the liver microsomal cytochrome P450 which, on using many of the more common techniques for solubilisation of membrane-bound enzymes, is converted into a metabolically inactive form called cytochrome P420, which possesses a reduced carbon monoxide spectrum with a Soret band at 420 nm (11). As the cholesterol-7α-hydroxylase contains cytochrome P450 as an essential component, it is necessary to retain the cytochrome in its active, P450, form during any solubilisation or fractionation technique.

Many techniques have been developed for the "solubilisation" of membrane bound enzymes, and several of these were employed in this study. They include the use of high molarity buffers, organic solvents, detergents, and enzymic digestion methods. Solubilisation is however an arbitrary term when applied to this type of system. The criterion used is an operational one of remaining in the supernatant after centrifugation at 105,000 g for

one hour. The density of the centrifugation medium and the particle preparation will both, therefore, have a bearing on whether a given procedure 'solubilises' the microsomes.

Lu and Coon (26) have developed one of the most successful methods for fractionation of liver microsomal mixed function oxidase enzymes. Their work has shown that there are at least three components necessary for the reconstitution of the enzyme namely a cytochrome P450 fraction, a flavoprotein with NADPH-oxidase activity, and a heat-stable lipid fraction. A lipid requirement for the 7α-hydroxylase has been investigated by use of organic solvents, and attempts have been made to apply the Lu and Coon method to the enzyme.

B. The Use of High Molarity Buffers

Conditions of high ionic strength are known to cause the dissociation of membrane systems giving a preparation which can be used for fractionation studies (43). Scholan used 1·0 M sodium phosphate buffer to dissociate a lyophilised preparation of microsomes and heat-treated 105,000 g supernatant (35). This preparation will remain suspended after centrifugation at 105,000 g for one hour, all the cholesterol 7α-hydroxylase activity remaining in the supernatant.

In this work either 1·0 M potassium phosphate or 2·0 M NaCl with 0·1 M sodium phosphate was used as the suspending medium. The use of sodium phosphate was discontinued due to the crystallisation of the buffer at low temperatures.

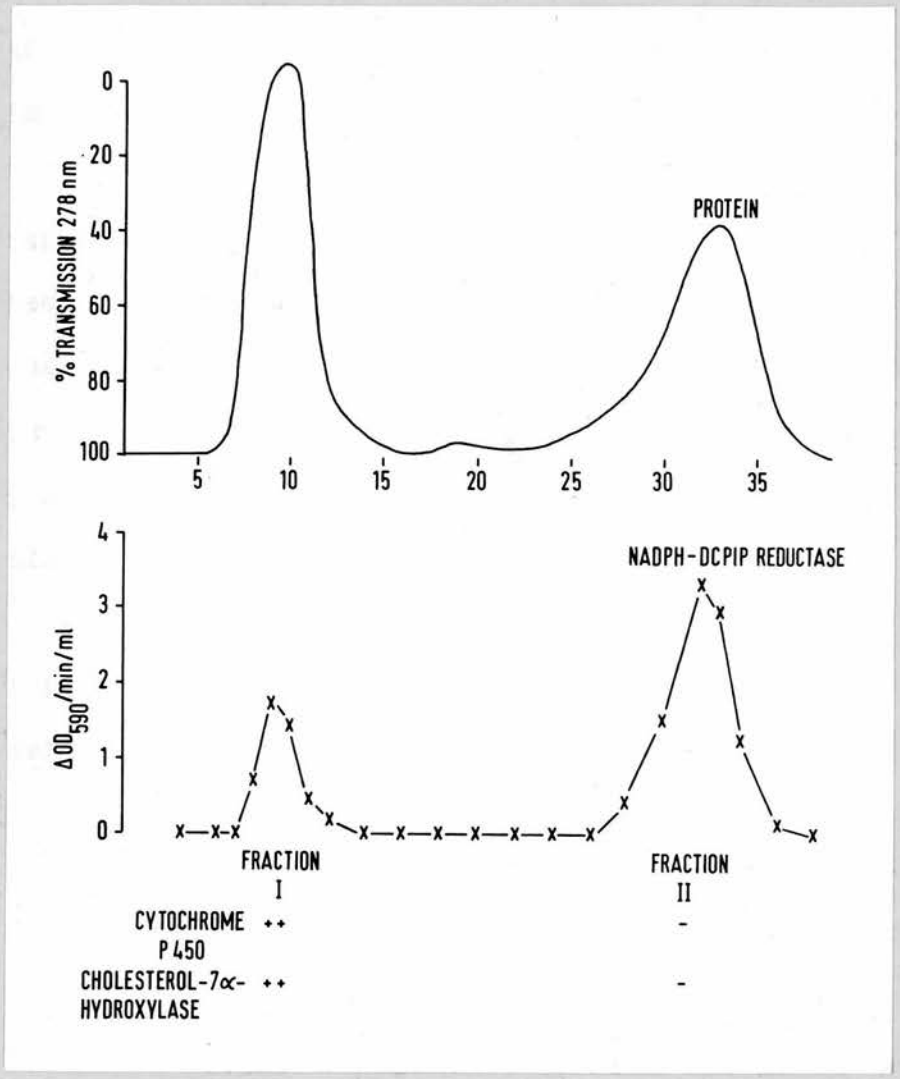

Figure 17. **The fractionation of 1 M phosphate buffer solubilised microsomes and heat-treated supernatant on a Sephadex G200 column.**

The upper graph shows the percentage transmission of the column effluent at 278nm.

The lower graph shows the NADPH-DCPIP reductase activity of each fraction collected.

The criterion of solubility of the enzyme preparation which is adopted is an arbitrary one. To obtain any fractionation of the microsomes, however, it is a minimum condition that the preparation remains in the supernatant after centrifugation at 105,000 g for one hour, these being the conditions under which untreated microsomes are precipitated.

A preparation of microsomes suspended in heat-treated 105,000 g supernatant and lyophilised was prepared, homogenised in buffered medium at high ionic strength at a concentration of 4 ml buffer per gram wet weight of liver, and centrifuged at 105,000 g for one hour. The entire cholesterol-7α-hydroxylase activity and the microsomal cytochrome P450 remained in the supernatant.

It was found to be necessary to homogenise the liver microsomes in the heat-treated 105,000 g supernatant in order to retain the maximal cholesterol-7α-hydroxylase activity. This is probably due to the antiperoxidative activity of the supernatant which protects the membrane lipids. If the heat-treated supernatant is omitted, large quantities of cytochrome P420 are found in the microsomes and much of the enzyme activity is lost.

Attempts were made with this preparation to obtain a separation of the microsomal components by gel filtration. A Sephadex G-200 column was used, equilibrated with 0·1 M phosphate buffer, pH 7·4. A trace of the absorption at 278 nm of the column effluent is shown in Figure 17. Two major components are obtained. The first fraction, which emerges at the void volume of the column

contains cytochrome P450, NADPH-DCPIP reductase activity and cholesterol 7α-hydroxylase activity. The other major peak, emerging at the total volume of the column contains NADPH-diaphorase activity. The first fraction, when centrifuged at 105,000 g for one hour gives a precipitate which contains the original enzymic properties of the microsomes, but in reduced amounts. It appears therefore that the dissociation induced by the high ionic strength is reversible and that reaggregation of the microsomes takes place on the Sephadex G-200 column. The second peak of protein obtained from the column is probably derived from the heat-stable 105,000 g supernatant, which consists mostly of low molecular weight material, and also has NADPH-DCPIP reductase activity.

Attempts were made to elute the column with 2·0 M NaCl buffered with phosphate, in order to prevent aggregation on the column, but it was found that the same elution pattern was obtained, due to slow reaggregation.

The use of buffers of high ionic strength to cause dissociation of the microsomes, therefore, is not suitable for use in fractionation studies, although a 'soluble' preparation of the microsomes can be obtained.

C. Extraction of the Microsomes with Organic Solvents

Many preparations of membrane bound enzymes involve the use of extraction with organic solvents (43). This step can remove much of the membrane lipid. This lipid creates a hydrophobic environment, and therefore increases the difficulty of

solubilisation in an aqueous medium. One of the most common organic solvent treatments is the preparation of an acetone precipitate. This operation is described fully in Section 2.

Preparation of an acetone precipitate of liver microsomes from cholestyramine-fed rats gives a preparation which will hydroxylate cholesterol in the 7α-position in the presence of 10 mM cysteamine with an activity greater than the native microsomes. This apparent increase in activity is probably due to the removal of membrane cholesterol by the acetone and ether washing procedure, thus causing less dilution of the radioactive tracer cholesterol added to the incubation medium. The acetone powder can be kept at -20° for at least one month without loosing its activity, and it is therefore a useful way of storing the enzyme.

Cholesterol-7α-hydroxylase activity is also retained in the liver microsomes after partitioning a homogenate of the microsomes with constant stirring for 10 minutes, with either ice-cold ether or with ice-cold iso-octane, as used in the method of Jefcoate et al. (78) in the preparation of soluble cholesterol side-chain cleavage enzyme from bovine adrenal mitochondria. Acetone, ether and iso-octane are all relatively apolar solvents.

If an acetone powder is made using acetone containing 10% methanol, however, no cholesterol-7α-hydroxylase activity is found in the resulting precipitate. The same is true if a liver microsomal homogenate is partitioned against n-butanol. Methanol and n-butanol are both relatively polar organic solvents. As all these solvents are removing various different lipids from the

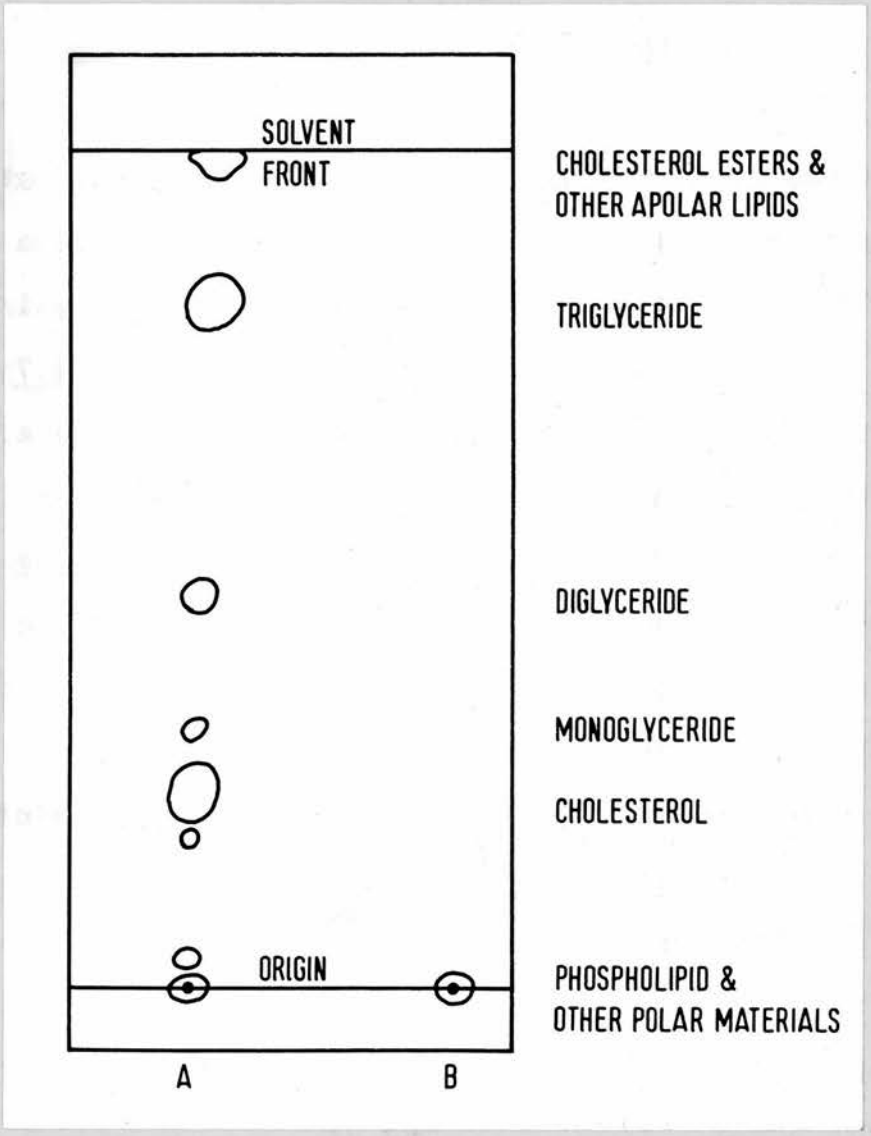

Figure 18. Identification of lipids (A) extracted with acetone/ether from the liver microsomes, and (B) remaining in the liver microsome acetone powder after extraction with acetone/ether. (TLC run on Silica Gel G with Hexane:Ether:acetic acid:: 75:24:1.)

microsomal membranes it should be possible to come to some conclusions concerning the lipid requirement of the cholesterol-7α-hydroxylase by analysing the lipids extracted into the organic solvent.

The total washings from an acetone-ether extraction of the liver microsomes were concentrated by evaporation under low pressure and analysed by TLC. The lipid analysis was carried out on Silica gel G using Hexane:Ether:acetic acid::75:24:1 as a solvent system. The lipids were visualised by spraying with sodium dodeca phosphomolybdate spray and heating in an oven for ten minutes. The lipids appear as dark spots against a yellow background. The identity of the various compounds was established by comparison of the Rf values of the spots with the Rf values of standard compounds in the same solvent system. The lipids found are identified in Figure 18. The acetone precipitate formed was extracted with chloroform:methanol 2:1, and the lipids present are identified in the same figure. It can be seen that only polar lipid materials, probably phospholipid, are present in active acetone powder. Further analysis of the phospholipid was carried out by TLC. The phospholipids were separated on Silica gel G containing a trace of sodium carbonate (95). The solvent system used to develop the plates was chloroform:methanol:acetic acid: water::50:25:6:4. The phospholipids present in the acetone powder were identified after visualisation with phosphomolybdate by comparison with standards and by comparison with published Rf values. No standard phosphatidyl inositol was available, and the

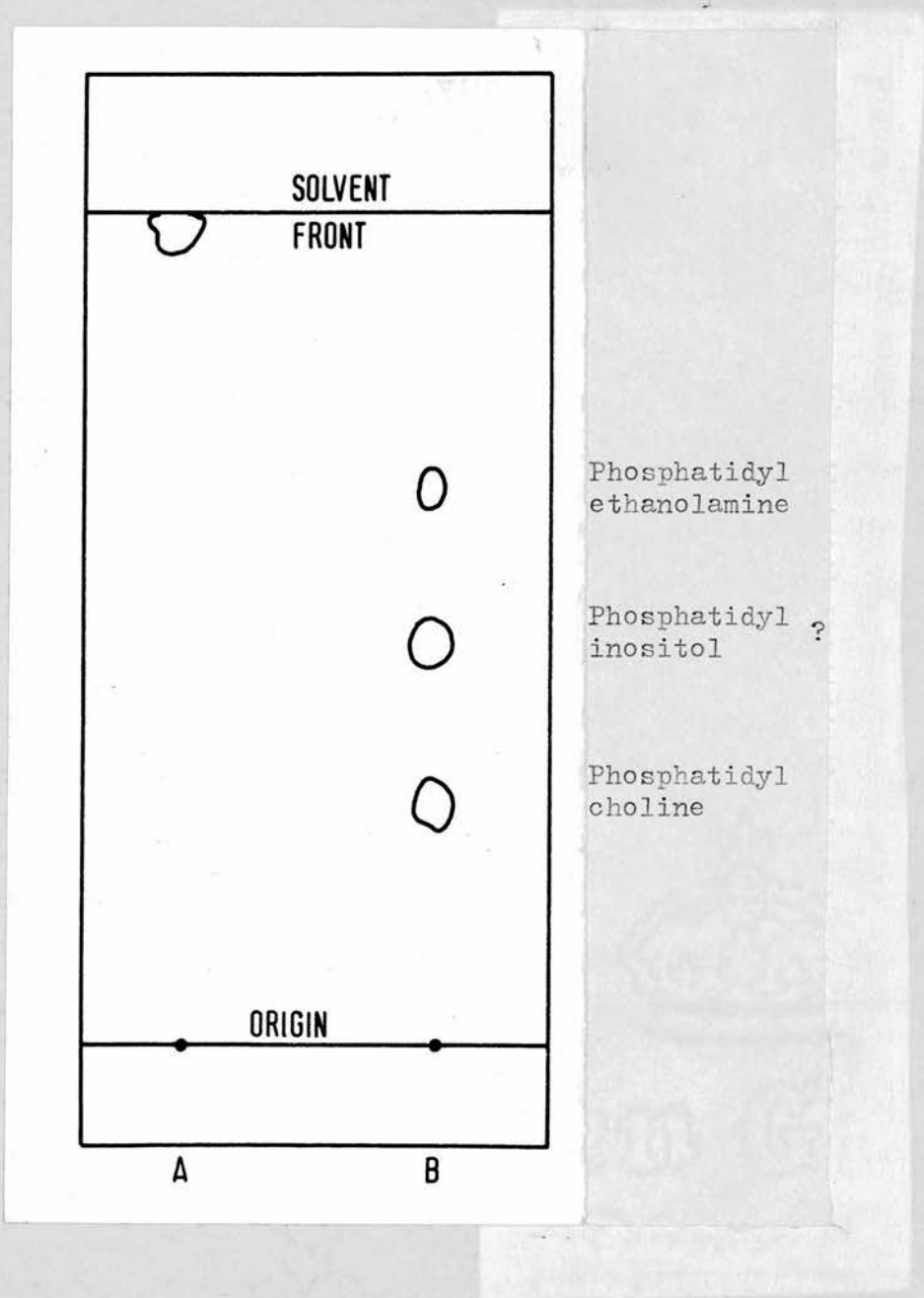

Figure 19. Identification of polar lipids (A) extracted with acetone/ether from liver microsomes, and (B) remaining in the liver microsomes acetone powder. (TLC run on Silica Gel G containing a trace of sodium carbonate with chloroform:methanol:acetic acid:water::50:25:6:4.)

identification of the spot as such in Figure 19 is on the basis of the published Rf only.

The origin material in Figure 18(A) extracted by the acetone-ether treatment did not correspond to any of the phospholipid standards, and remains unidentified.

If 10% methanol is included in the acetone when preparing the acetone precipitate, no cholesterol-7α-hydroxylase activity is retained. This mixture is found to extract much of the phospholipid material from the microsomes. Imai and Sato (44) found that aniline hydroxylase activity could be recovered in similar circumstances by adding back the extracted lipid to the acetone/methanol powder. This procedure did not restore a significant amount of cholesterol hydroxylating ability to the methanol/acetone powder, probably due to the large amounts of cholesterol present in the extract, which would dilute the radioactive tracer to a very large extent. It is also possible that the acetone/methanol extraction destroys the cholesterol-7α-hydroxylase, although this is unlikely as intact cytochrome P450 is present in the methanol/acetone powder, which also retains NADPH-DCPIP reductase activity. It seems reasonable therefore to assume that, like the situation described by Coon et al. (18), the phospholipid is vital for the activity of the mixed function oxidase enzyme.

The acetone powder is not a suitable material for use in fractionation of the microsomes, as treatment with solubilising agents such as detergents and high molarity buffers causes rapid

conversion of the P450 into the P420 form. This occurs at a far greater rate than in the native microsomes, suggesting that the lipid which has been extracted by the acetone treatment, although not vital for enzymic activity, has a stabilising effect. It has been found that the isolation of the cytochrome P450 from the camphor oxidase system of Pseudomonas putida is greatly facilitated by the presence of the substrate camphor, which stabilises the cytochrome P450 and prevents conversion to the P420 form (76). It is also found that the soluble form of cytochrome P450 from the adrenal cortex mitochondria which catalyses the side-chain cleavage of cholesterol is isolated in the form of an enzyme-substrate complex (93). It is likely that the reason for the instability of the cholesterol-7α-hydroxylase of acetone powder to solubilization techniques is the removal of the substrate cholesterol as demonstrated above.

D. The Use of Proteases

Limited digestion of membrane systems has been used in several instances to detach membrane bound proteins (35,45). It has been especially useful in the preparation of electron transport components from the liver microsomes, cytochrome b_5 and NADPH-cytochrome c reductase both having been obtained in this manner. Proteolytic digestion has been used to prepare an insoluble cytochrome P450 preparation from the liver microsomes which is uncontaminated with other cytochromes and therefore very useful in spectral studies (20). This preparation is still attached to the

particulate portion of the microsomes.

An attempt was made, using Subtilisin (Sigma Protease Type VII) to carry out a proteolytic 'dissection' of the microsomes and to reconstitute the 7α-hydroxylase enzyme system.

Microsomes from cholestyramine-fed rats were homogenised in 0·1 M phosphate buffer containing 25% glycerol at a protein concentration of 5 mg per ml. Subtilisin was added at a concentration of 0·75 mg per ml and the mixture was incubated for 18 hours under an atmosphere of nitrogen. After the incubation, the mixture was diluted with 0·1 M phosphate buffer to twice the volume in order to reduce the viscosity, and centrifuged at 105,000 g. Table 4 shows the amounts of cytochrome P450, cytochrome b_5, and NADPH-cytochrome c reductase found in the pellet and supernatant. A very good separation of the first and second components of the microsomal mixed function oxidase electron transport system is found. This separation has useful characteristics for a preliminary step in a purification procedure for the enzyme components. However, although this proteolytic digestion can be used for the preparation of the NADPH-cytochrome c reductase and of cytochrome P450 particles separately, it is found that, on recombination of the two components, no aminopyrine demethylase activity, or cholesterol 7α-hydroxylase activity is present.

It appears therefore that the digestion process has destroyed some part of the enzyme system which allows the interaction of the cytochrome P450 and the flavoprotein. Coon et al. (46) have also

Table 4.

The separation of cytochromes P450 and b_5, NADPH-cytochrome c reductase and protein between the particulate and soluble fractions after digestion of the rat liver microsomes with Subtilisin.

	Supernatant concentration	Precipitate concentration	% of total in supernatant	% of total in precipitate
P450	0·01 nmoles/ mg protein	0·35 nmoles/ mg protein	2	98
b_5	1·13 nmoles/ mg protein	0·09 nmoles/ mg protein	90	10
NADPH-cyt c reductase	0·260 (ΔOD_{550} min/mg protein)	0·019 (ΔOD_{550} min/mg protein)	91	9
Protein	-	-	42	58

found that the NADPH-cytochrome c reductase flavoprotein prepared by this method does not interact with the fractionated P450 preparation from a DEAE cellulose column.

The proteolytic digestion of the rat liver microsomes can therefore be used for studying the components of the mixed function oxidase electron transport chain, but cannot be used for reconstitution studies.

E. The Use of Detergents

Of all the methods so far described, the use of detergents is probably the most widely applied method for the solubilisation of membrane bound enzyme systems. (See for examples ref (43)). Among the more commonly used detergents are bile salts, and synthetic compounds of the Lubrol, Triton, and Tween series. Sodium deoxycholate and Lubrol WX have both been applied with some success to cytochrome P450 containing enzymes of the liver microsomes and to the cytochrome itself (46,47).

It has been found necessary when using detergent solubilisation methods to utilise glycerol to hinder the conversion of the cytochrome P450 to P420. Ichikawa and Yamano have shown that glycerol can reverse this process and reconvert cytochrome P420 into P450 (48). The solubilisation method of Lu and Coon (26) utilises deoxycholate as a detergent, glycerol, KCl to maintain the ionic strength, and dithiothreitol as a sulphydryl protecting agent in citrate buffer.

The Lu and Coon method was applied to microsomes isolated from cholestyramine-fed rats as follows. The microsomes were suspended

in 0·25 M sucrose at a concentration of 30 mg of protein per ml of solution. The protein suspension was then mixed with a solution of 24 ml glycerol, 8 ml 1·0 M potassium citrate buffer pH 7·6, 8 ml 1·0 M KCl and 0·8 ml 0·10 M cysteamine, in a ratio of 1 ml of suspension to 1 ml of the solution. A solution of 10% sodium deoxycholate was then added with constant stirring to the mixture to a concentration of 1 mg sodium deoxycholate per 2·5 mg of microsomal protein. After 10 minutes stirring, the mixture was centrifuged at 105,000 g for two hours. A soluble cytochrome P450 preparation is obtained, but there is no cholesterol-7α-hydroxylase activity present in the supernatant. This is probably due to the inhibition of the enzyme by deoxycholate, as shown by Scholan (35). As the deoxycholate is necessary for the solubilisation procedure, and the subsequent column chromatography, a method of removing the deoxycholate for assay purposes was devised.

The preparation was passed through a 50 ml Sephadex G25 column equilibrated with 10% glycerol in 0·1 M phosphate buffer. The protein is eluted in the void volume, and the deoxycholate is retarded. The protein preparation from the Sephadex G25 column carries out the 7α-hydroxylation of cholesterol, thus demonstrating that the enzyme can be solubilised by the Lu and Coon method.

Having obtained a solubilised preparation, which, after treatment to remove the deoxycholate, will hydroxylate cholesterol at the 7 position, attempts were made to fractionate the microsomes on DEAE cellulose, duplicating the separation method of Lu and Coon, using a scaled-down procedure. A 3 x 30 cm column of Sigma DEAE

cellulose preequilibrated with 0·1 M tris-HCl buffer containing 0·05% sodium deoxycholate and 0·2mM cysteamine was used. The soluble microsomal preparation (460 mgs microsomal protein) was diluted to 3 times its volume with water and applied to the column. The column was washed with 0·1 M tris-HCl buffer containing 0·05% sodium deoxycholate. A stepwise gradient of KCl in 0·1 M steps was applied to the column, and the fractions were collected. The fractions were monitored for protein by measurement of the transmission of light at 278 nm, for cytochromes P450 and b_5, and for NADPH-DCPIP reductase activity. A fraction containing cytochrome P450 and P420 was eluted with 0·2 M KCl. The cytochrome b_5 is eluted with 0·3 to 0·4 M KCl. No separation was obtained with the NADPH-DCPIP reductase, which appeared in both the cytochrome b_5 and P450 fractions.

Removal of deoxycholate from these fractions by the method already described, and recombination of the fractions did not result in reconstitution of the cholesterol-7α-hydroxylase activity. It is probable that the enzyme was permanently inactivated due to prolonged exposure to sodium deoxycholate. G25 chromatography of the P450 fraction to remove the deoxycholate resulted in reconversion of a substantial amount of the cytochrome P420 to the P450 form. This is an illustration of the phenomenon noted by Ichikawa and Yamano (48), who showed that glycerol could reconstitute cytochrome P450 from P420 formed by treatment of the microsomes by deoxycholate.

It is probable that using larger amounts of microsomal protein

and therefore larger, faster flowing columns that a successful fractionation of the cholesterol-7α-hydroxylase enzyme could be accomplished. Studies of this nature by Boyd et al. have also been unsuccessful so far (92). It is possible however that the failure recorded here is a genuine result and that the cholesterol-7α-hydroxylase is not amenable to this type of fractionation, unlike the drug-hydroxylating enzymes with which the method has been successful.

A second detergent fractionation method which was attempted involved the use of ammonium sulphate fractionation of a sodium chclate solubilized preparation of microsomes. This was carried out as follows. The liver microsomes prepared from cholestyramine-fed rats were suspended in 0·1 M phosphate buffer at a protein concentration of 10-12 mg per ml. Sodium cholate was added to the suspension to a concentration of 0·5 mgs per mg protein, with constant stirring. This resulted in an opalescent pink suspension. Neutralised saturated ammonium sulphate solution containing 10 mg per ml sodium cholate was then added to bring the concentration of ammonium sulphate to 15% of saturation. The protein precipitate was removed by centrifugation and resuspended and dialysed against 0·1 M sodium phosphate buffer pH 7·4 containing 1 mM cysteamine and 10% glycerol. The dialysis was used to remove the contaminating cholate and ammonium sulphate. This process was repeated on the 15% ammonium sulphate saturated supernatant taking fractions at 30%, 40%, and 60% ammonium sulphate saturation.

After discarding the 0 - 15% fraction due to precipitation and turbidity, each dialysed fraction was analysed for total protein, cytochromes P450 and b_5 concentration, cholesterol-7α-hydroxylase and aminopyrine demethylase activity, n-octylamine difference spectrum and degree of solubilization as judged by centrifugation. These results are presented in Table 5. It can be seen in the table that there is little separation of, for example, cytochrome P450 from cytochrome b_5, nor is there any change in the ratio of high- to low-spin cytochrome P450 as judged by the n-octylamine method, the ratio remaining at approximately 1:3 high- to low-spin throughout the fractionation. However, in the 40% - 60% fraction (F4) cholesterol-7α-hydroxylase activity, aminopyrine demethylase activity, substrate binding difference spectra and cytochromes P450 and b_5 have been retained. In addition F4 is not precipitated by centrifugation at 250,000 g for one hour. It therefore has all the characteristics of a good soluble preparation of the microsomes, in the presence of only trace amounts of detergent.

Attempts were made to fractionate this preparation using a DEAE cellulose column method identical to that previously described for the Lu and Coon procedure, except that 0·05% sodium deoxycholate was omitted from the eluant. The protein remained bound to the DEAE cellulose however at a concentration of 0·7 M KCl, and it therefore appears that this method of fractionation is unusable.

Table 5.

The ammonium sulphate fractionation of sodium cholate solubilized rat liver microsomes.

Fraction ($(NH_4)_2SO_4$ % saturation)	Appearance of suspension	Total protein mg	P450 nmoles/ mg protein	b_5 nmoles/ mg protein
F_1 (0 - 15%)	very turbid and white	-	-	-
F_2 (15 - 30%)	opalescent	22·5	0·33	0·21
F_3 (30 - 40%)	slightly cloudy, red	63·5	0·43	0·23
F_4 (40 - 60%)	clear, deep red	85·0	0·32	0·42

	cholesterol-7α-hydroxylase % conversion	Aminopyrine demethylase nmoles/min/mg protein	n-octylamine $(OD_{\lambda max} - OD_{\lambda min})$/mg protein
F_1	-	-	-
F_2	0·85	0·06	0·0055
F_3	1·19	0·04	0·0061
F_4	2·66	0·19	0·0096

	Precipitation
F_1	Precipitates rapidly on standing
F_2	Precipitates slowly on standing
F_3	Precipitates at 105,000 g for 1 hour
F_4	"Soluble" after 250,000 g for 1 hour

SUMMARY OF RESULTS

1) A soluble preparation of microsomes can be prepared with the use of high-molarity phosphate buffer, but due to reaggregation, fractionation cannot be accomplished.

2) Microsomes treated with apolar organic solvents retain cholesterol-7α-hydroxylase activity, whereas microsomes treated with more polar organic solvents lose cholesterol-7α-hydroxylase activity. Analysis of the extracted lipids shows that phospholipid is necessary for NADPH-cytochrome P450 reduction to take place. Removal of the apolar lipids from the microsomal membranes renders the cholesterol-7α-hydroxylase activity and the cytochrome P450 highly labile to treatment with solubilising agents.

3) Limited digestion of the microsomes with Subtilisin gives a soluble preparation of NADPH-cytochrome c reductase and a membrane bound P450 preparation. No reconstitution of cholesterol-7α-hydroxylase or NADPH-cytochrome P450 reductase activity occurs, due to loss of interaction between the two components of the electron transport chain.

4) Application of the Lu and Coon procedure to microsomes from cholestyramine-fed rats results in a soluble preparation of the microsomes, which, on removal of the deoxycholate by Sephadex G25 chromatography retains cholesterol-7α-hydroxylase activity. DEAE-cellulose chromatography of the soluble preparation results in separation of the cytochrome P450 from cytochrome b_5. Reconstitution of the cholesterol-7α-hydroxylase activity was not successful.

5) Ammonium sulphate fractionation of sodium cholate solubilized microsomes gives soluble microsomal preparation, but no separation of components.

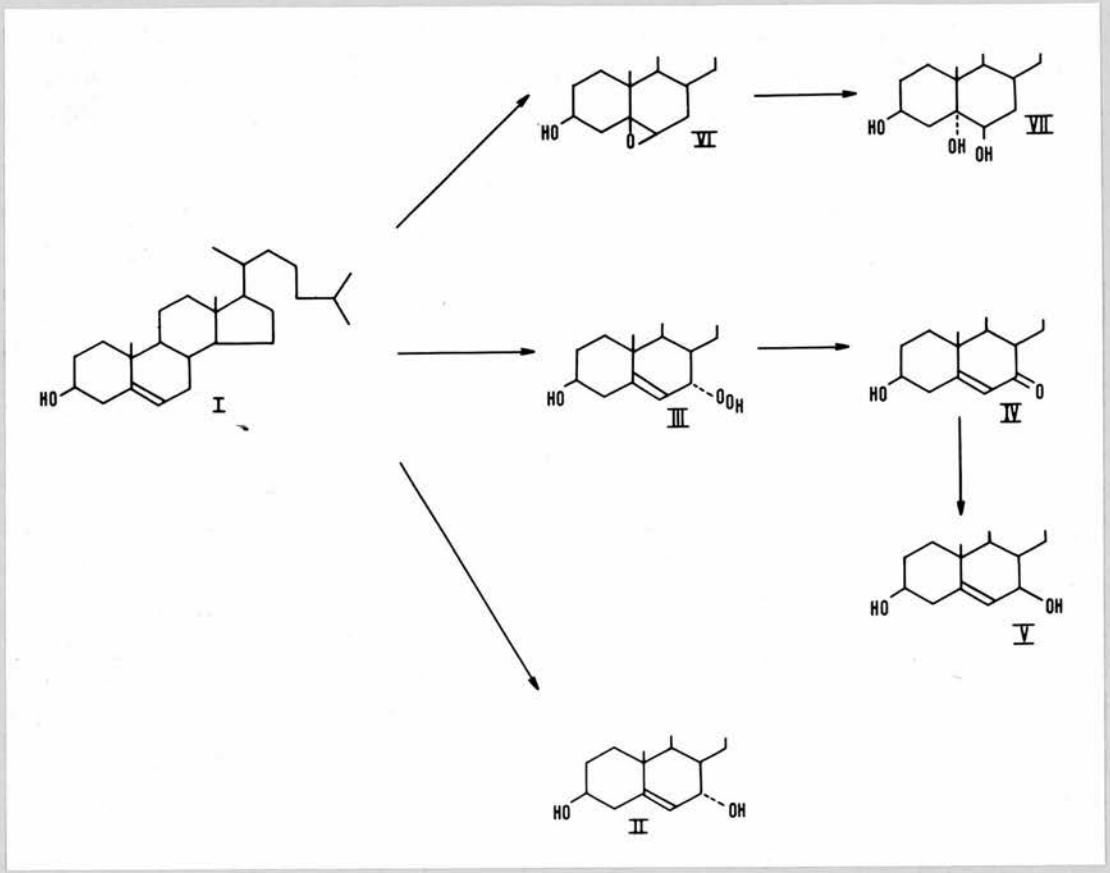

Figure 20. Possible mode of formation of cholesterol autoxidation products (From Mitton et al. (5)). The products are (I) cholesterol, (II) 7α-hydroxycholesterol, (III) cholesterol-7α-hydroperoxide, (IV) 7-ketocholesterol, (V) 7β-hydroxycholesterol, (VI) cholesterol-5,6-oxide, (VII) cholestan-3β, 5α,6β,-triol.

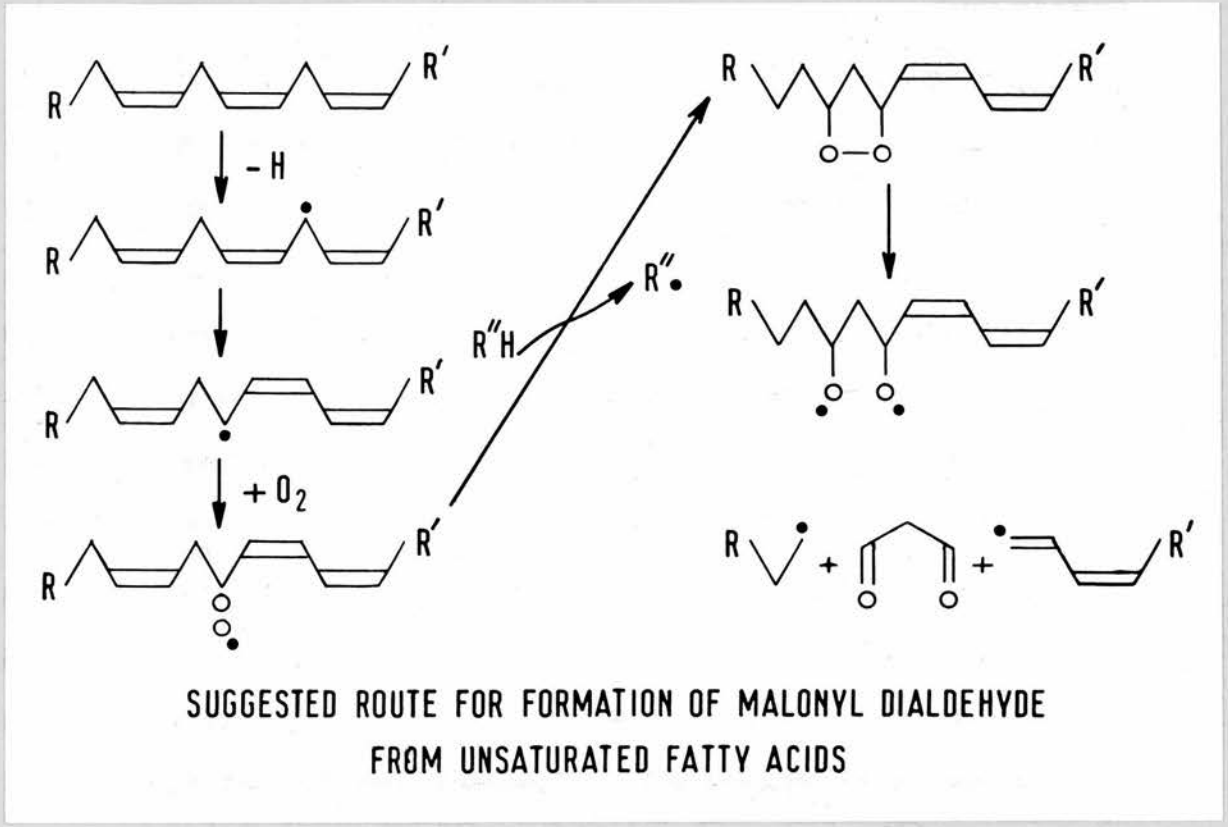

Figure 21. Proposed mode of formation of malonaldehyde from the peroxidation of unsaturated fatty acids in the liver microsomes. (After Dahle et al. (50)).

SECTION 5

THE PEROXIDATION OF CHOLESTEROL AND OTHER LIPIDS IN THE RAT LIVER MICROSOMES.

A. Introduction

The incubation of rat liver microsomes with (4-^{14}C)-cholesterol in the presence of NADPH and oxygen, as described in Section 2 gives rise to a series of radioactive products which have been identified in this and other laboratories (5,49). These products are known as "autoxidation products" and they are thought to arise from non-physiological oxidations of the cholesterol molecule. The main autoxidation product of cholesterol formed in the liver microsomes is 7-keto-cholesterol, which can be reduced in the microsomes to 7β-hydroxycholesterol. The mode of formation of these and other autoxidation products of cholesterol as proposed by Mitton et al. (5) is shown in Figure 20. Cholesterol is not the only lipid material known to undergo this type of oxidation. The unsaturated fatty acids of the microsomal membranes are peroxidised giving rise to malonaldehyde (50) (See Fig. 21). The peroxidation of fatty acids is also dependent on the presence of NADPH. This Section is concerned with an investigation of the characteristics of cholesterol autoxidation and fatty acid peroxidation, and of the factors which affect the rate of each. It seems likely that the oxidations are two manifestations of the same phenomenon, namely, the non-physiological attack of oxygen in the presence of NADPH on the oxidisable components of the liver microsomal membranes.

The peroxidation of unsaturated fatty acids is measured by the

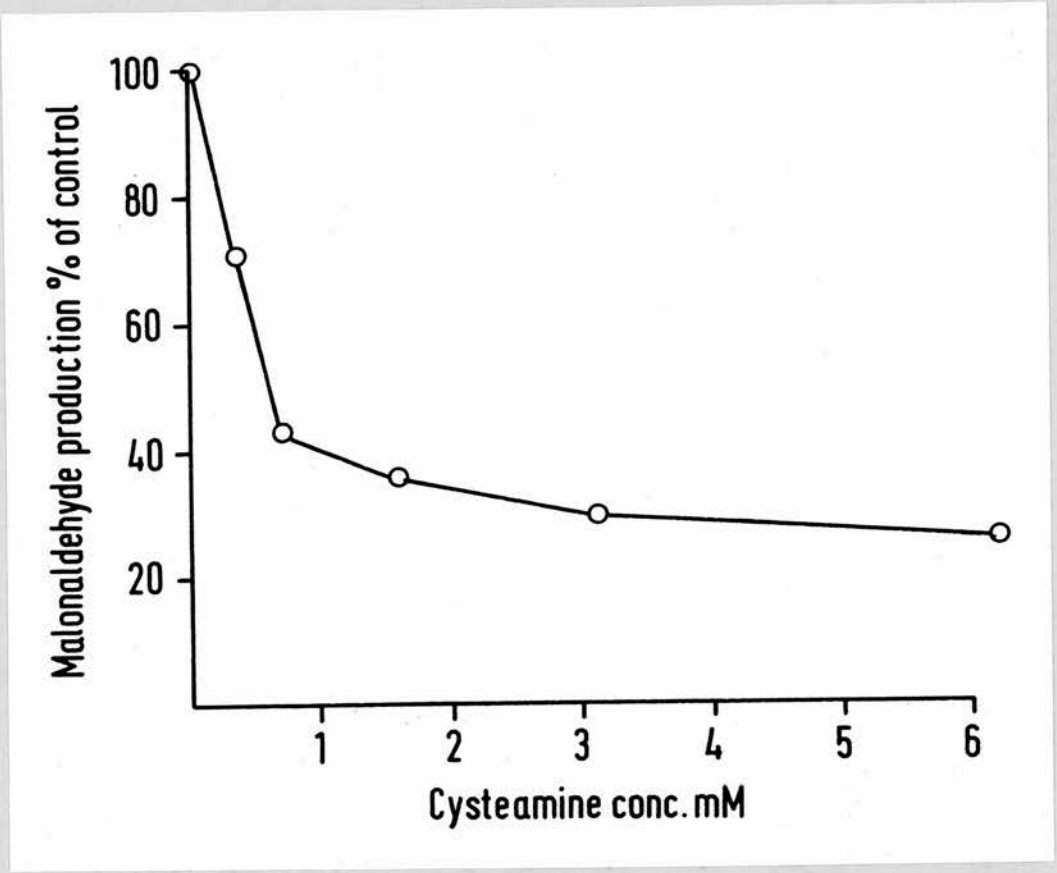

Figure 22. The effect of 8-mercaptoethylamine on the production of malonaldehyde from the liver microsomes in the presence of NADPH.

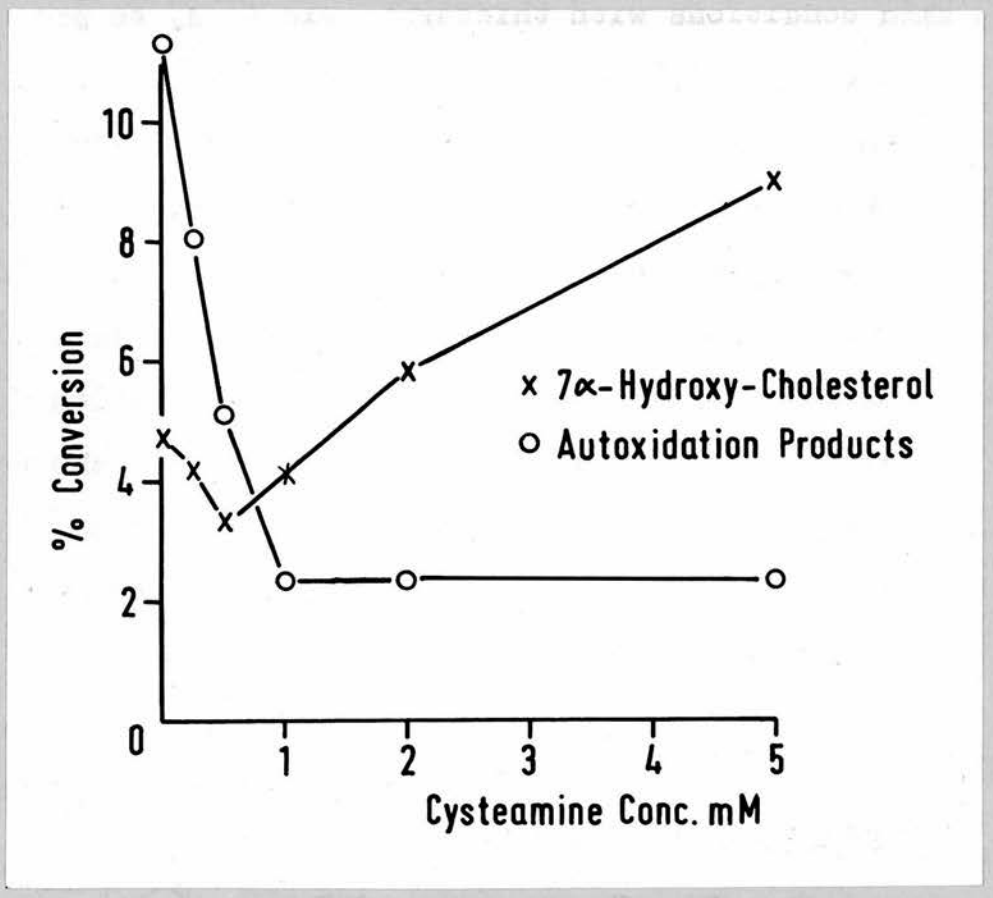

Figure 23. The effect of β-mercaptoethylamine on the oxidation of cholesterol in liver microsomes.

formation of malonyl dialdehyde. It is measured by condensation in acid conditions with thiobarbituric acid, to produce a red colour, which is measured spectrophotometrically.

Total oxidation is assayed by oxygen uptake, measured by use of a Clarke oxygen electrode. A certain amount of the NADPH stimulated oxygen uptake is due to hydroxylation of endogenous substrates such as fatty acids, but this is quantitatively very small compared with the uptake due to peroxidation.

Mitton, Scholan and Boyd have shown that cholesterol oxidation is stimulated by the presence of ADP and ferrous ions (5), in common with lipoperoxidation as demonstrated by Hochstein and Ernster (28).

B. The Effect of β-mercaptoethylamine on Malonaldehyde production and Cholesterol Oxidation

Figures 22 and 23 show the effect of β-mercaptoethylamine (cysteamine) on the production of malonaldehyde and the oxidation of cholesterol. There is a strong inhibition of both cholesterol autoxidation and malonaldehyde production, both being inhibited by 50% at 0·4 - 0·6 mM cysteamine. It should be noted however that the concentrations of cysteamine given are those present at the start of the assay incubations, and that the concentration of cysteamine, as measured by thiol concentration, drops markedly throughout the incubation period.

With regard to the production of 7α-hydroxycholesterol, it can be seen that this is minimal up to a concentration of 1 mM

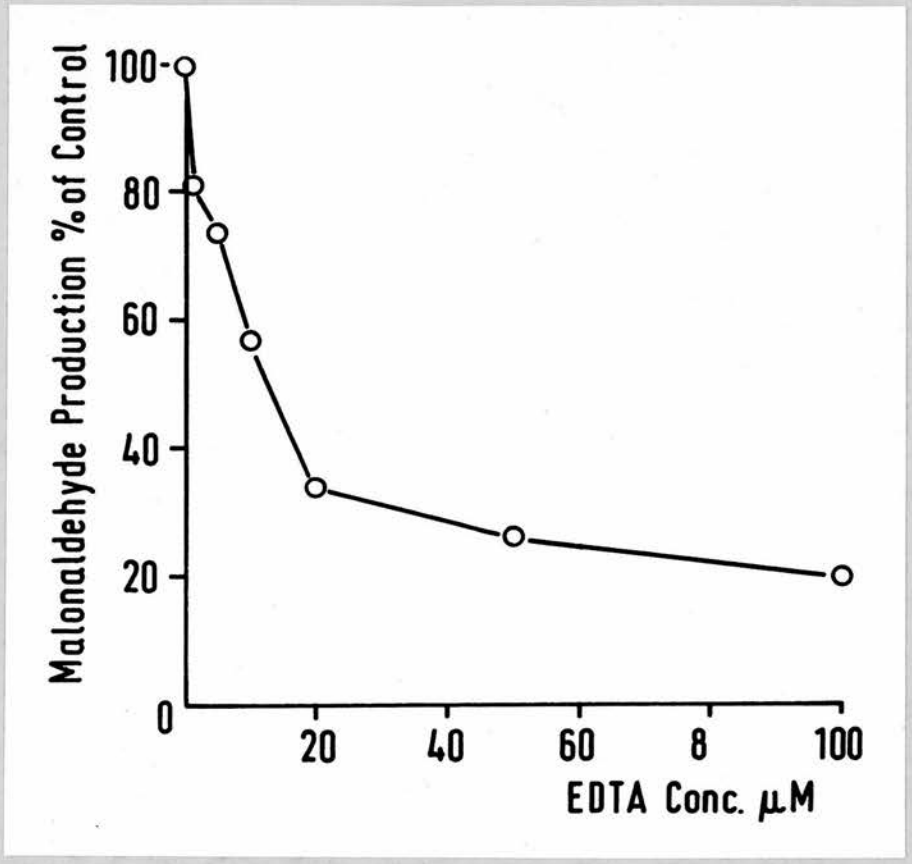

Figure 24. The effect of EDTA on the production of malonaldehyde in liver microsomes.

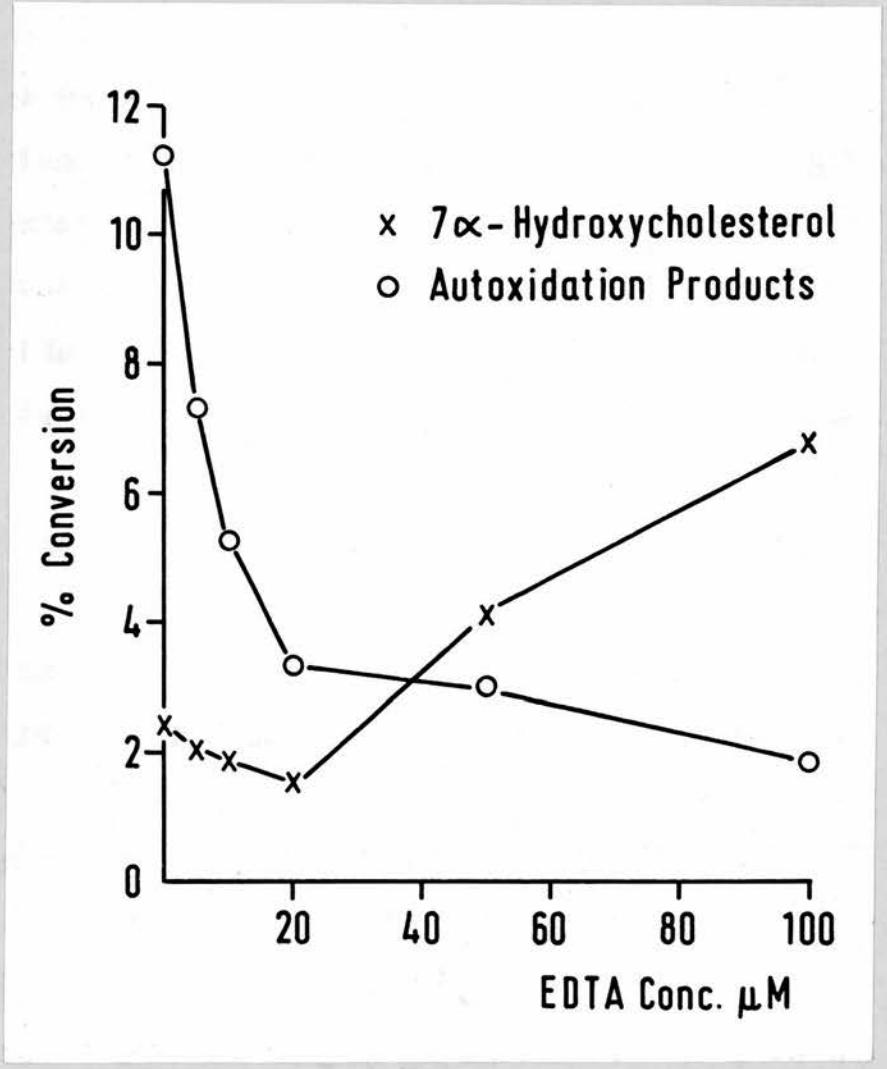

Figure 25. The effect of EDTA on the oxidation of cholesterol in liver microsomes.

cysteamine at which concentration the formation of autoxidation products is minimal. At concentrations of greater than 1 mM cysteamine, however, there is marked stimulation of the 7α-hydroxycholesterol. At concentrations greater than 10 mM there is inhibition of 7α-hydroxycholesterol formation by cysteamine (35).

Figure 5 in Section 2 shows examples of radioactive scans of thin layer chromatography separations of the products of incubation of (4-^{14}C)cholesterol with liver microsomes in the presence and the absence of 10 mM cysteamine, showing complete inhibition of all cholesterol oxidation products except for 7α-hydroxycholesterol.

C. The Effect of EDTA on Malonaldehyde Production and Cholesterol Oxidation

EDTA, a powerful metallic ion chelator is known to have an inhibitory effect on lipid peroxidation (51). Figures 24 and 25 show the effect of EDTA at various concentrations on malonaldehyde production and cholesterol oxidation in the liver microsomes. The results obtained are very similar to those for cysteamine, 50% Inhibition of cholesterol autoxidation and lipid preoxidation is obtained at a concentration of 10 - 12 μM EDTA. Stimulation of 7α-hydroxycholesterol production occurs above a concentration of 20 μM at which maximal inhibition of the formation of autoxidation products has been accomplished.

Other chelators, such as des-ferrioxamine and sodium diethyldithiocarbamate also inhibit the autoxidation of cholesterol, as demonstrated by Scholan (35).

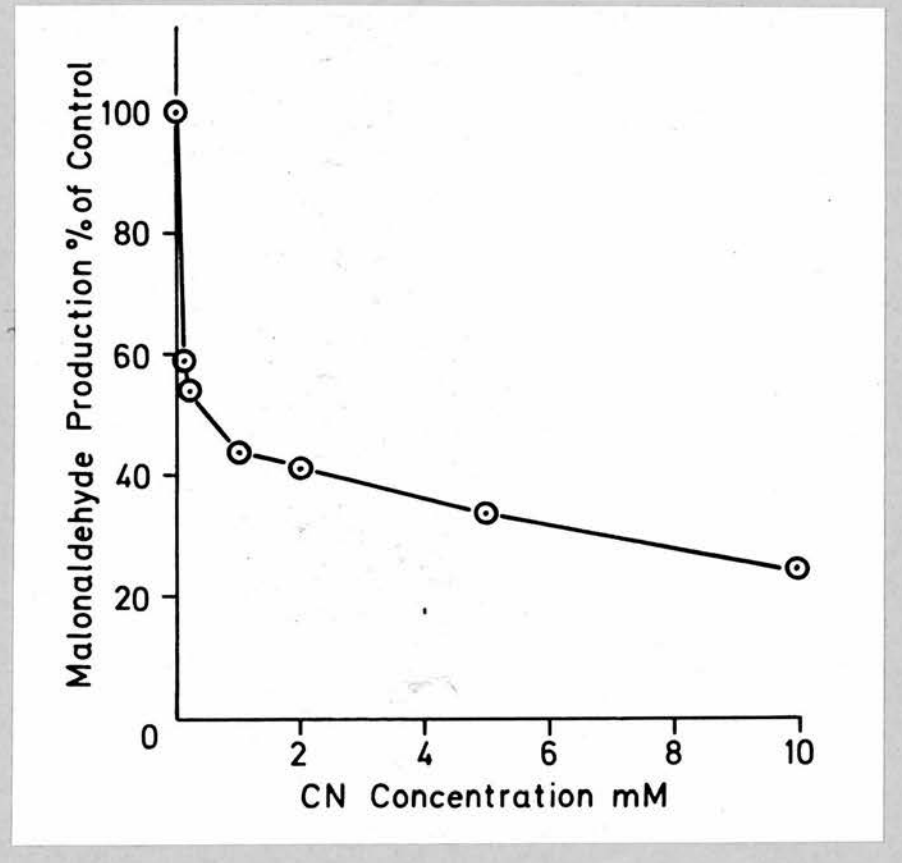

Figure 26. The effect of cyanide ions on the production of malonaldehyde in liver microsomes.

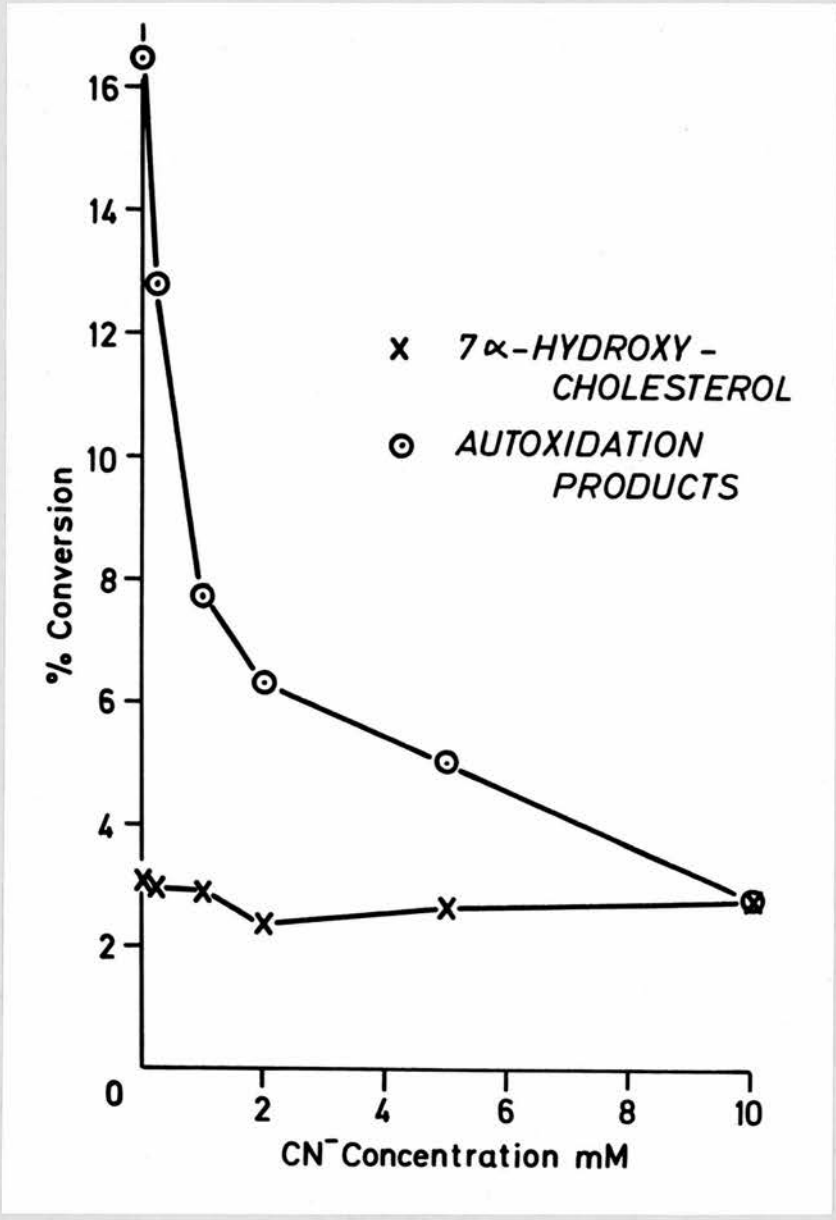

Figure 27. The effect of cyanide ions on cholesterol oxidation in liver microsomes.

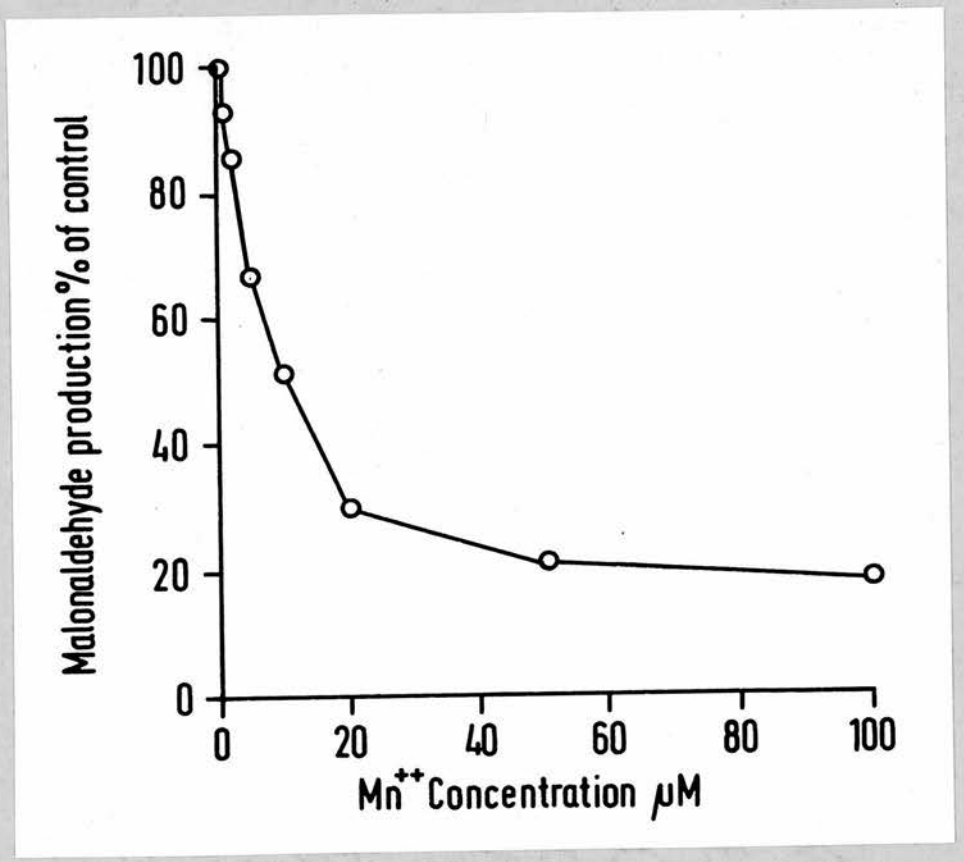

Figure 28. The effect of manganous ions on the production of malonaldehyde in liver microsomes.

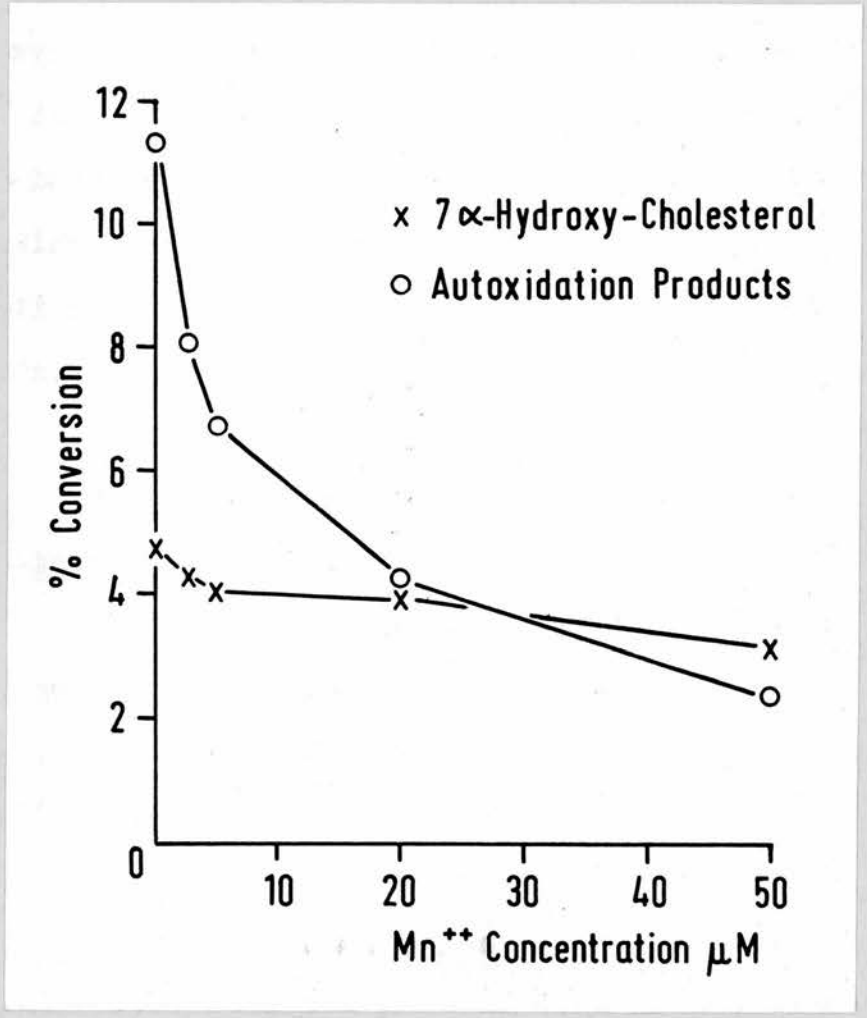

Figure 29. The effect of manganous ions on cholesterol oxidation in liver microsomes.

D. The Effect of Cyanide Ions on Malonaldehyde Production and Cholesterol Oxidation

Cyanide ions were found to have an inhibitory effect on the production of malonaldehyde and cholesterol autoxidation products as shown in Figures 26 and 27. The fact that cyanide does not cause inhibition of cholesterol-7α-hydroxylase at these concentrations in conditions where no autoxidation products are formed, leads one to suspect that the reason for its inhibition of oxidation reactions of a non-specific nature is its chelating ability, rather than inhibition by interference at the enzyme active site.

E. The Effect of Metal Ions on Malonaldehyde Production and Cholesterol Autoxidation

As chelating agents have a marked effect on lipid peroxidation the effect of various metal ions was tested. As has already been noted, ferrous ions, especially in the presence of ADP, have a marked stimulatory effect on the production of autoxidation products of cholesterol and of malonaldehyde (28, 5). This stimulatory property is shared with cadmium and zinc ions. Several metal ions have very little effect. This group includes nickel and calcium. Of greatest interest are the metal ions which cause an inhibition of cholesterol autoxidation and malonaldehyde production. The most effective of this group is the manganous ion. The effect of manganous ions on malonaldehyde production and cholesterol oxidation is shown in Figures 28 and 29. Cobaltous ions and then chromous

ions are next in efficiency of inhibition. It is interesting to note in Figure 29 that, unlike inhibitors of cholesterol autoxidation such as cysteamine and EDTA, (but like cyanide ions), the production of 7α-hydroxycholesterol does not rise as the production of autoxidation products is inhibited. It therefore seems that the inhibitors of cholesterol autoxidation are of two types, those which stimulate and those which do not stimulate cholesterol-7α-hydroxylation at higher concentrations. Cysteamine and EDTA have a dual effect of inhibiting autoxidation and promoting cholesterol-7α-hydroxylase, whereas cyanide and manganous ions have no stimulatory effect on cholesterol-7α-hydroxylase. This phenomenon is discussed further in Section 8.

SUMMARY OF RESULTS

1) Cholesterol autoxidation and lipid peroxidation in the liver microsomes require the presence of NADPH and oxygen.

2) Both reactions are inhibited by β-mercaptoethylamine.

3) Both reactions are inhibited by EDTA.

4) Both reactions are inhibited by cyanide ions.

5) Both reactions are inhibited by manganous and cobaltous ions and stimulated by ferrous ions.

6) The conclusion is that lipid peroxidation and cholesterol autoxidation are different aspects of the same general phenomenon, and affected by the same factors.

SECTION 6

FACTORS AFFECTING CHOLESTEROL AUTOXIDATION 'IN VIVO'

A. Introduction

Having established that cholesterol autoxidation and lipid peroxidation as measured by oxygen uptake and malonaldehyde production are probably two manifestations of the same process, namely oxygen attack in a non-specific manner on the oxidisable components of the liver microsomes, it is of great interest to investigate the means whereby these peroxidation reactions are kept under control *in vivo*. Peroxidation reactions are highly deleterious to cell membrane systems which contain large quantities of polyunsaturated fatty acids and cholesterol, both of which are easily peroxidised. It is known that in several pathological conditions such as carbon tetrachloride poisoning and radiation damage peroxidation occurs with drastic results (52, 53). Peroxidation of amino acids, proteins, carbohydrates and nucleic acids can also occur in these pathological conditions. As NADPH and oxygen are present at all times in the liver, it seems likely that peroxidation may occur due to an overloading or breakdown of some peroxidation inhibiting system.

Mitton (54) showed that incubation of the mitochondrion-free 18,000 g supernatant of rat liver with NADPH, O_2 and radioactive tracer cholesterol resulted in the formation of 7α-hydroxy cholesterol only, whereas incubation of the microsomes under the same conditions gave rise to autoxidation products of cholesterol. It is possible therefore that the peroxidation inhibiting system

is present in the cell supernatant.

Christophersen (55) put forward the theory that lipid peroxidation is inhibited by the action of an enzyme, Glutathione peroxidase, which catalyses the reaction between a lipid hydroperoxide and reduced glutathione as follows.

$$ROOH + 2GSH \longrightarrow ROH + GSSG + H_2O$$

This gives rise to a harmless hydroxy compound, and removes a hydroperoxide, which could form free radicals and react with other lipid molecules in an autocatalytic manner, causing aberrant oxidation products to be formed.

However, it has been shown in this laboratory that the rat liver supernatant retains its anti-lipoperoxidative activity even after heating on a boiling water bath for ten minutes, treatment which would destroy the heat labile glutathione peroxidase enzyme (56).

Another theory, of which Tappel is a main proponent, is that Vitamin E is the main biological lipid antioxidant, pointing to evidence from Vitamin E deficiency studies, and to the powerful in vitro inhibition of lipid peroxidation by α-tocopherol (57). The possibility of the involvement of Vitamin E in the anti-lipoperoxidative activity of the heat-treated supernatant of rat liver has been tested.

As the heat treated supernatant has the same effect on cholesterol autoxidation as the untreated supernatant, this section is concerned with the investigation of the heat treated supernatant and its effect on cholesterol autoxidation, oxygen uptake and

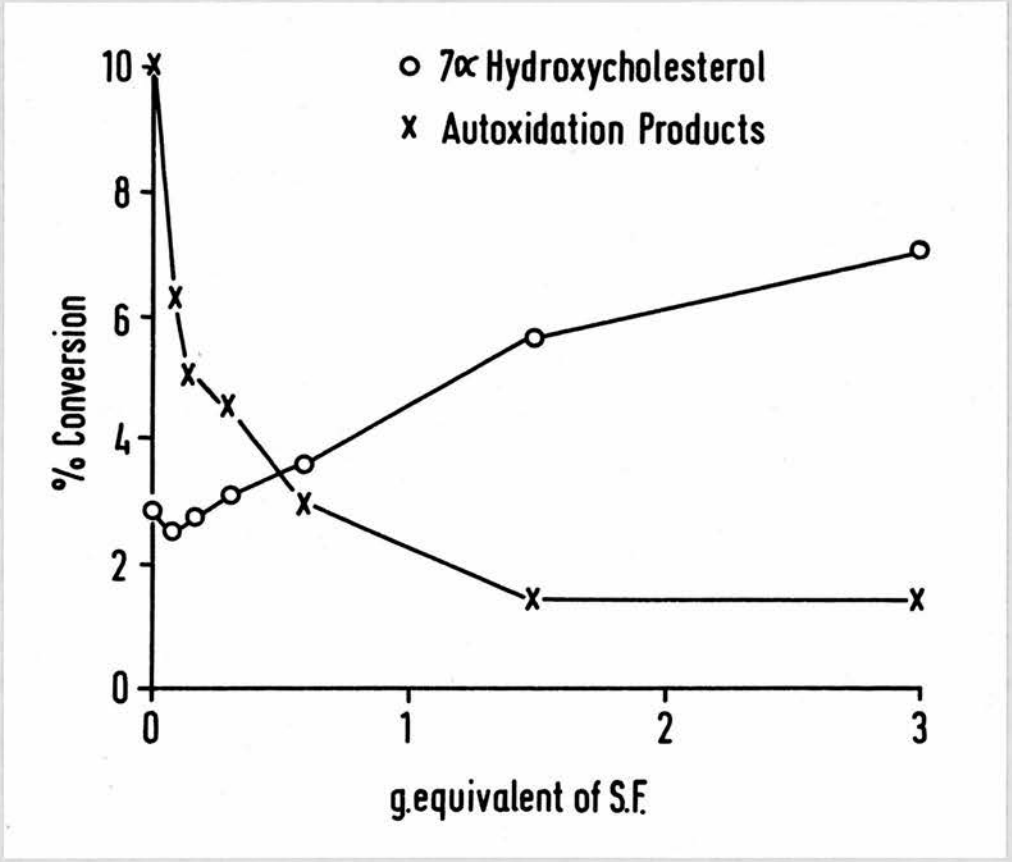

Figure 30. The effect of heat-treated liver supernatant on cholesterol oxidation in liver microsomes. A g. equivalent of S.F. is the amount of S.F. obtained from one gram wet weight of liver.

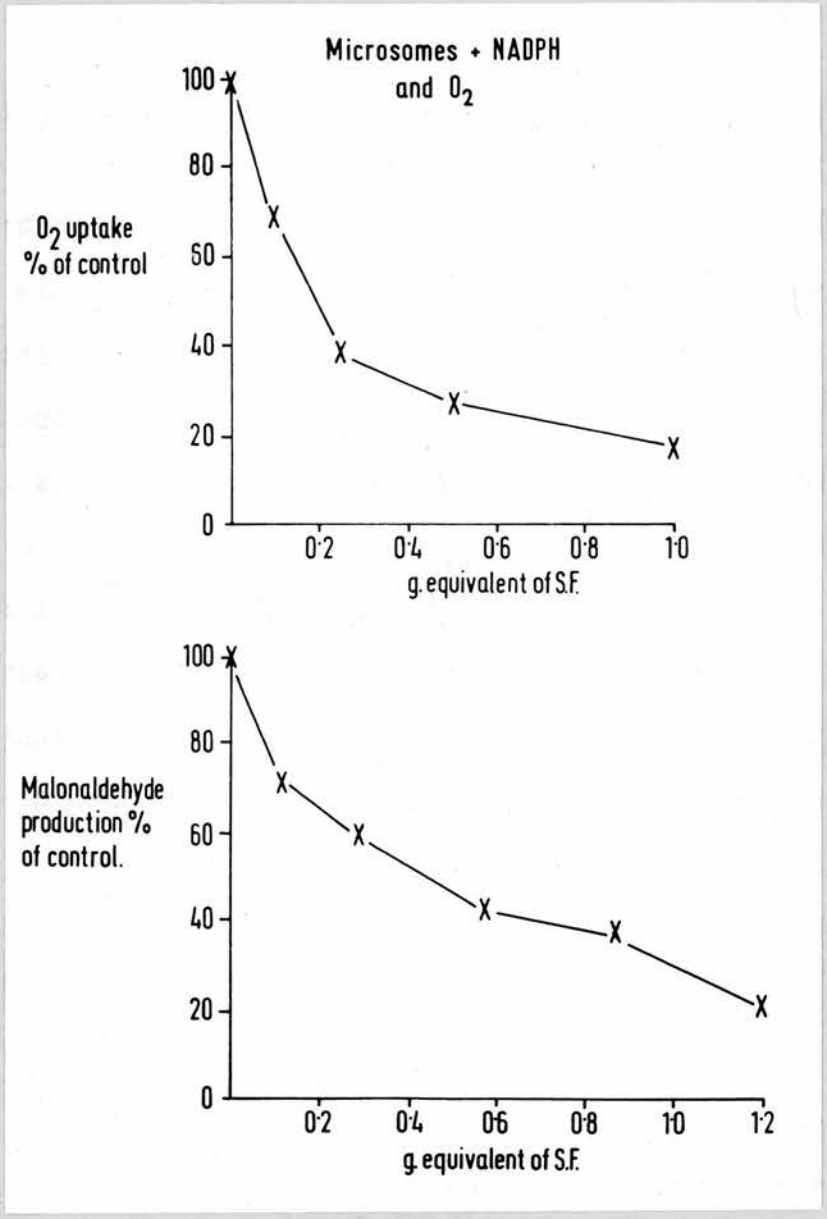

Figure 31. The effect of heat-treated liver supernatant on oxygen uptake and malonaldehyde production of liver microsomes.

A g. equivalent of S.F. is the amount of S.F. obtained from one gram wet weight of liver.

malonaldehyde production by the rat liver microsomes.

B. The Effect of the Heat-Treated Supernatant of Rat Liver on Peroxidation Reactions

The effect of varying amounts of heat-treated supernatant on cholesterol oxidation is shown in Figure 30. As can be seen, there is a marked inhibition of cholesterol autoxidation at lower concentrations then stimulation of the production of 7α-hydroxy cholesterol at higher concentrations. There is a remarkable similarity between Figure 30 and Figures 23 and 25 which showed the effect of cysteamine and EDTA on cholesterol oxidation.

Figure 31 shows the effect of the heat-treated supernatant on the oxygen uptake and the malonaldehyde production from the liver microsomes incubated aerobically in the presence of NADPH. A marked inhibition is again obtained.

The inhibition obtained in all these assays of peroxidative activity is very close to that obtained with the untreated 105,000 g supernatant, suggesting that the essential physiological anti-peroxidative system is heat stable. The soluble heat-stable factor or factors have been designated as S.F.

C. The Effect of Prolonged Heating on the S.F.

Figure 32 shows the malonaldehyde production from rat liver microsomes, in the presence of fixed amounts of supernatant which have been heated in a boiling water bath for varying amounts of time. Small portions of supernatant were used in order to facilitate rapid

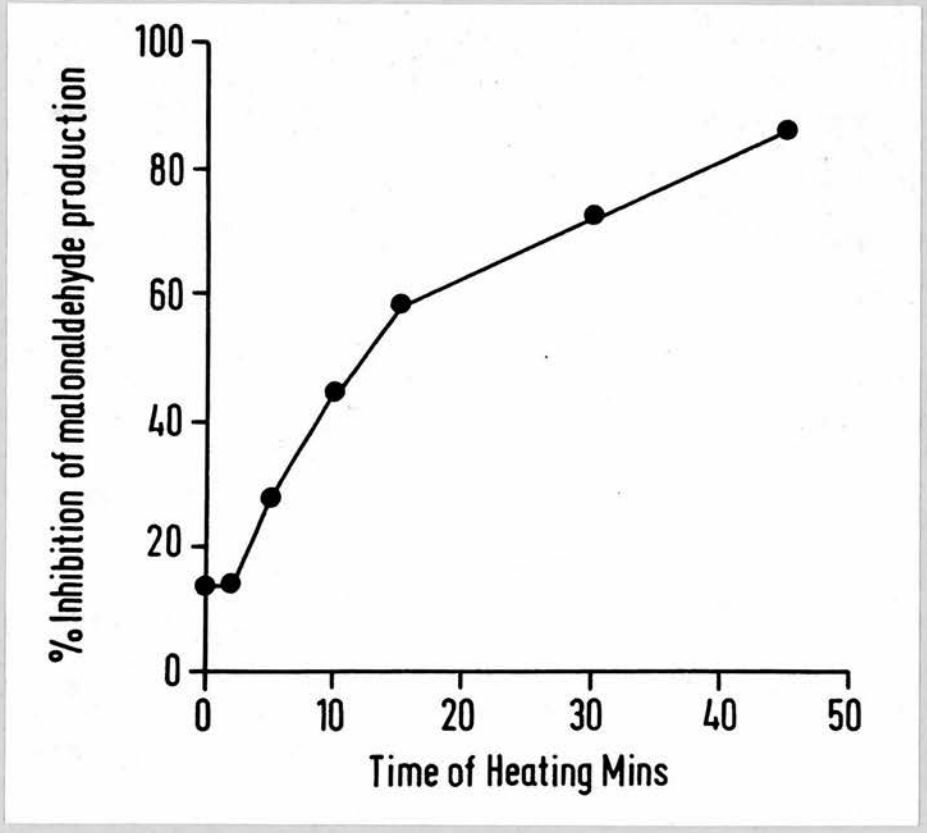

Figure 32. The effect of prolonged heating of the S.F. on the inhibition of malonaldehyde production in liver microsomes.

heating. All protein coagulation appears to take place within the first two minutes of heating. As can be seen in the figure, after two minutes heating the supernatant has the same effect on malonaldehyde production as the native supernatant. After this time, activity is gradually lost, suggesting that some process other than protein denaturation is responsible for the loss in activity.

D. The Effect of Lipid Extraction on the S.F.

Repeated partitioning of heat-treated supernatant against ether or chloroform causes no diminution of the effectiveness of the S.F. in inhibiting the production of malonaldehyde from the liver microsomes. This suggests that the active component of the S.F. is not lipid in nature. This experiment excludes the possibility that the active component of the S.F. is vitamin E or a derivative. Tappel has suggested that vitamin E is the main anti-lipoperoxidative factor *in vivo* (57).

E. The Effect of Dialysis on S.F.

S.F. was dialysed against 0·154 M KCl for various times and its efficiency at inhibiting malonaldehyde production was compared with S.F. which had been stored undialysed. The results are shown in Table 6, all inhibitions being given as a percentage of the control value. As can be seen, the efficiency of the S.F. at inhibiting malonaldehyde production is markedly diminished by dialysis as compared

Table 6

The effect of dialysis on the S.F.

Time of dialysis Hours	Malonaldehyde production Percentage of control
0	23
6	38
18	79
36	96

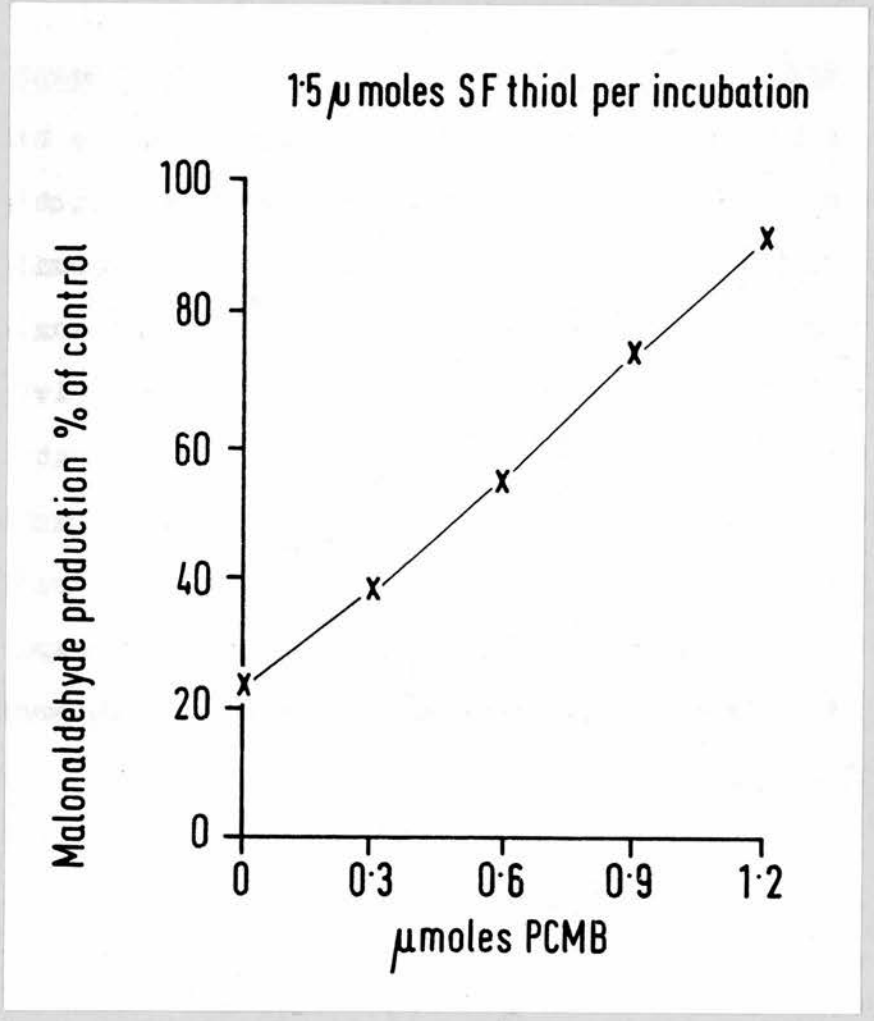

Figure 33. The effect of pCMB pretreatment of the S.F. on the inhibition of malonaldehyde production in liver microsomes.

with non-dialysed S.F. This seems to suggest that one of the components of the anti-peroxidative system is of a low molecular weight, and is therefore unlikely to be an enzyme.

F. The Effect of p-Chloromercuribenzoate Treatment on the S.F.

It has already been shown that cysteamine has a potent inhibitory effect on the lipid peroxidation reactions of the rat liver microsomes, and several other thiol compounds are known to exert a modifying action on the cholesterol autoxidation reactions (58). The possibility that a thiol was an active component of the S.F. was therefore investigated. The concentration of thiol in the heat-treated supernatant as measured by Ellman's reagent was found to be 3 - 4 μmoles per gram wet weight of rat liver. Aliquots of S.F. containing 5 μmoles of thiol were added to incubation flasks, and varying amounts of p-chloromercuribenzoate (pCMB), up to 3 μmoles were added to the flasks. After ten minutes shaking in order to allow all the pCMB to react with the thiol, a microsomal suspension and an NADPH generating system were added to the flasks in the usual manner and a malonaldehyde assay was carried out after 20 minutes. The result is shown in Figure 33. As the thiol concentration drops in the incubation medium by reaction with the pCMB, the amount of malonaldehyde produced increases, tending towards maximal production of malonaldehyde when all the thiol in the S.F. has been titrated out. A similar result is obtained if the same experiment is repeated with the cholesterol-7α-hydroxylase

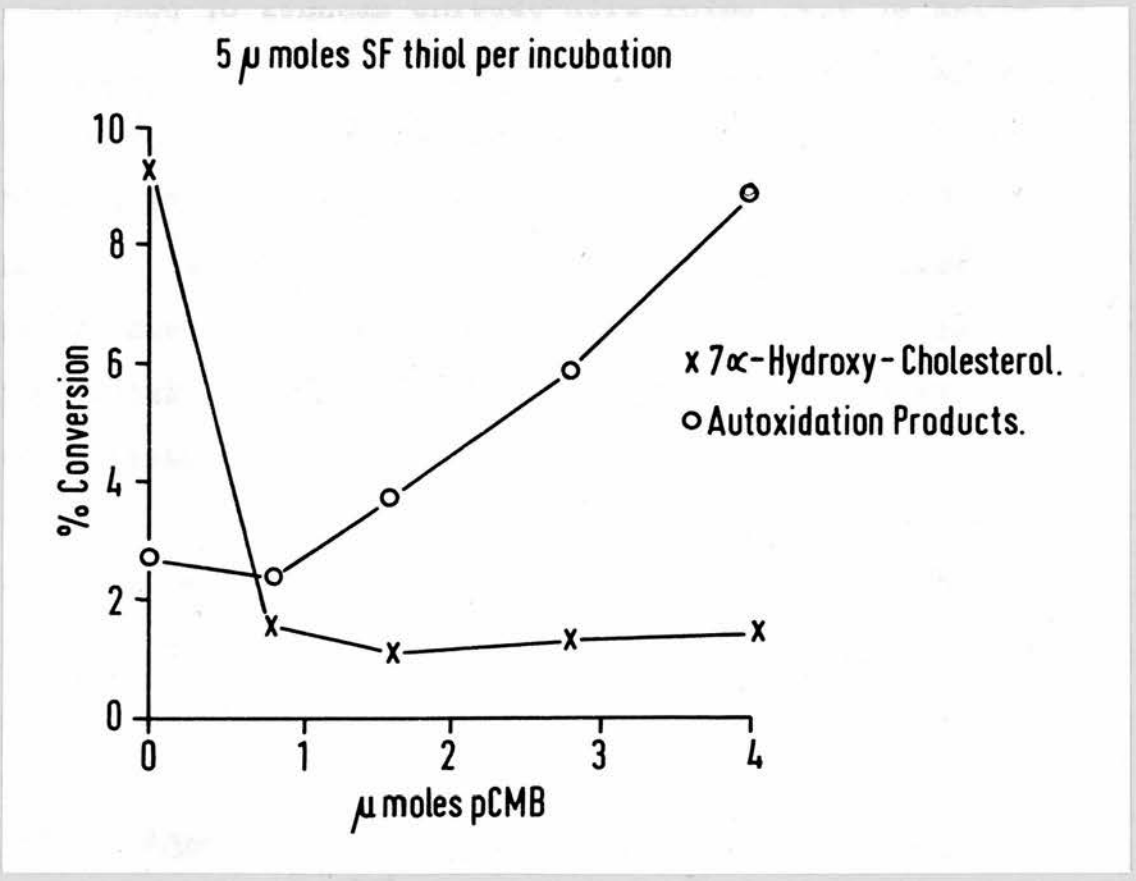

Figure 34. The effect of pCMB pretreatment of the S.F. on the inhibition of cholesterol oxidation in liver microsomes.

assay system. Figure 34 shows the effect of pretreating 5 μmoles of S.F. thiol with varying amounts of pCMB and then carrying out the assay incubations. As the concentration of thiol in the incubation drops, the production of 7α-hydroxycholesterol also drops. At low concentrations of thiol, the production of autoxidation products of cholesterol increases. This graph is an inverted form of the pattern obtained with increasing concentration of cysteamine (Figure 23). These experiments indicate that the majority of the thiol groups in the S.F. is necessary for the action of the peroxidation-inhibiting system.

The next step in the investigation was to identify the thiol components present in the heat-stable portion of the rat liver supernatant.

G. Fractionation of the S.F. by Exclusion Chromatography

Before column fractionation procedures could be attempted, a method of concentrating the heat-treated supernatant was needed. Lyophilisation of the S.F. immediately after precipitation of the denatured protein by centrifugation was found to preserve most of its inhibitory activity. The pinkish-yellow powder obtained could be stored at -20° for several weeks without loss of activity and it was this preparation that was used for testing various column fractionation procedures. It was decided to use a gel filtration method for a preliminary fractionation as this type of column can be used for bulk preparation with great ease and reproducibility. G-25 Sephadex eluted with water was found to give a separation into

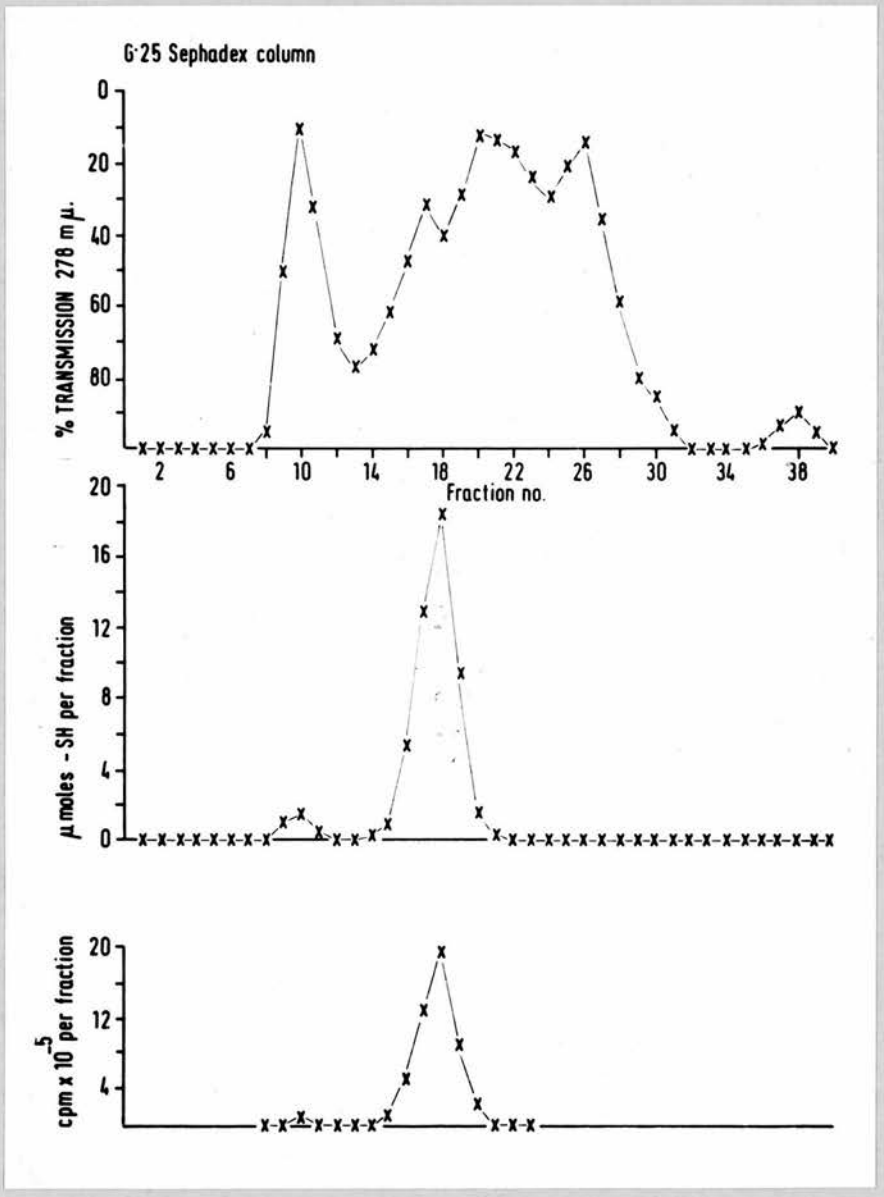

Figure 35. The Sephadex G25 chromatography of heat-treated liver supernatant from rats pretreated with (^{35}S) cysteine.

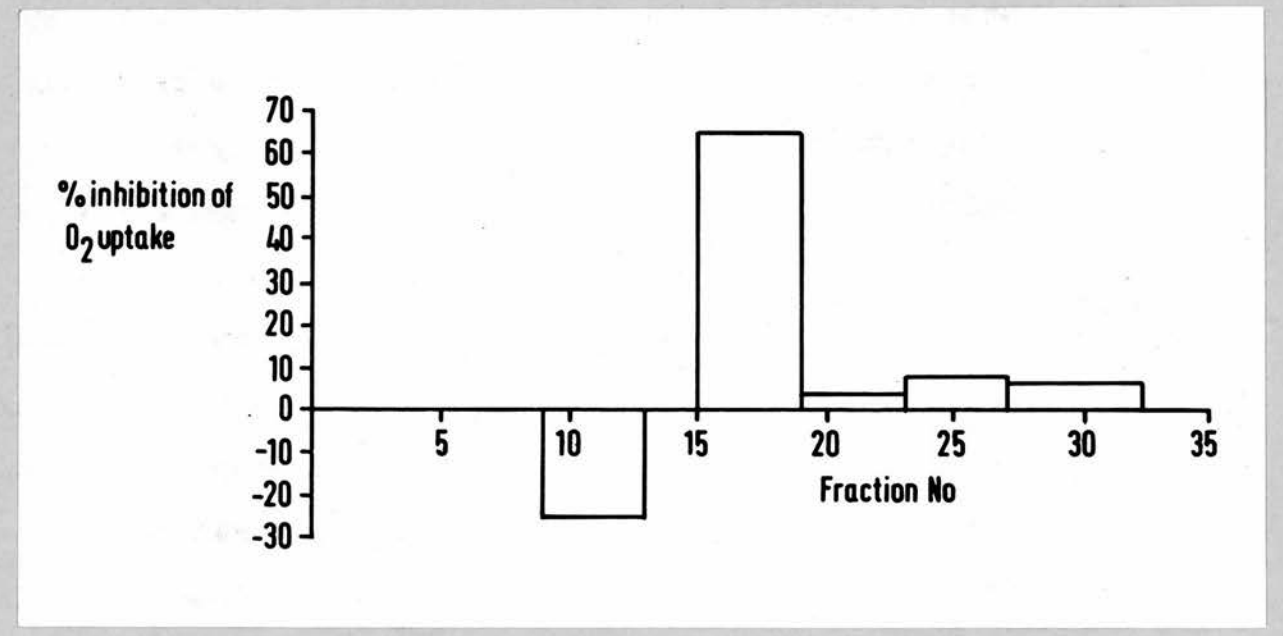

Figure 36. Effect of pooled fractions from Sephadex G25 chromatography of heat-treated supernatant of rat liver on NADPH-stimulated oxygen uptake of rat liver microsomes.

a wide range of products, and this type of column was used in all subsequent experiments.

As one of the components to be identified was known to be a thiol, the experimental rats were injected with ^{35}S labelled cysteine (From The Radiochemical Centre, Amersham, 20 mCi/mmole.) 30 minutes before killing. A delay time of 30 minutes was found to give the maximum incorporation of radioactive label into the S.F. The S.F. was prepared as usual and the lyophilised preparation was applied to a G-25 Sephadex column and eluted with water. The result is shown in Figure 35, the column effluent being monitored for absorption at 278 nm to show the presence of protein and also nucleotides, thiol groups and ^{35}S radioactivity. As can be seen there is only one main thiol component eluted from the column, which coincides with the one main radioactive band. The effect of the various fractions from the column, when pooled and lyophilised, on the NADPH stimulated O_2 uptake of the liver microsomes is shown in Figure 36, demonstrating that only the thiol cut from the column has an inhibitory activity on lipid peroxidation. This is further demonstrated by the fact that the thiol fraction from the G-25 column, when compared with the heat-treated supernatant, with respect to their relative efficiencies in inhibiting malonaldehyde production from the microsomes, has the same efficiency of inhibition when compared on the basis of thiol concentration in the incubation medium. This gives rise to the conclusion that the entire anti-lipoperoxidative system is preserved intact throughout the exclusion chromatography.

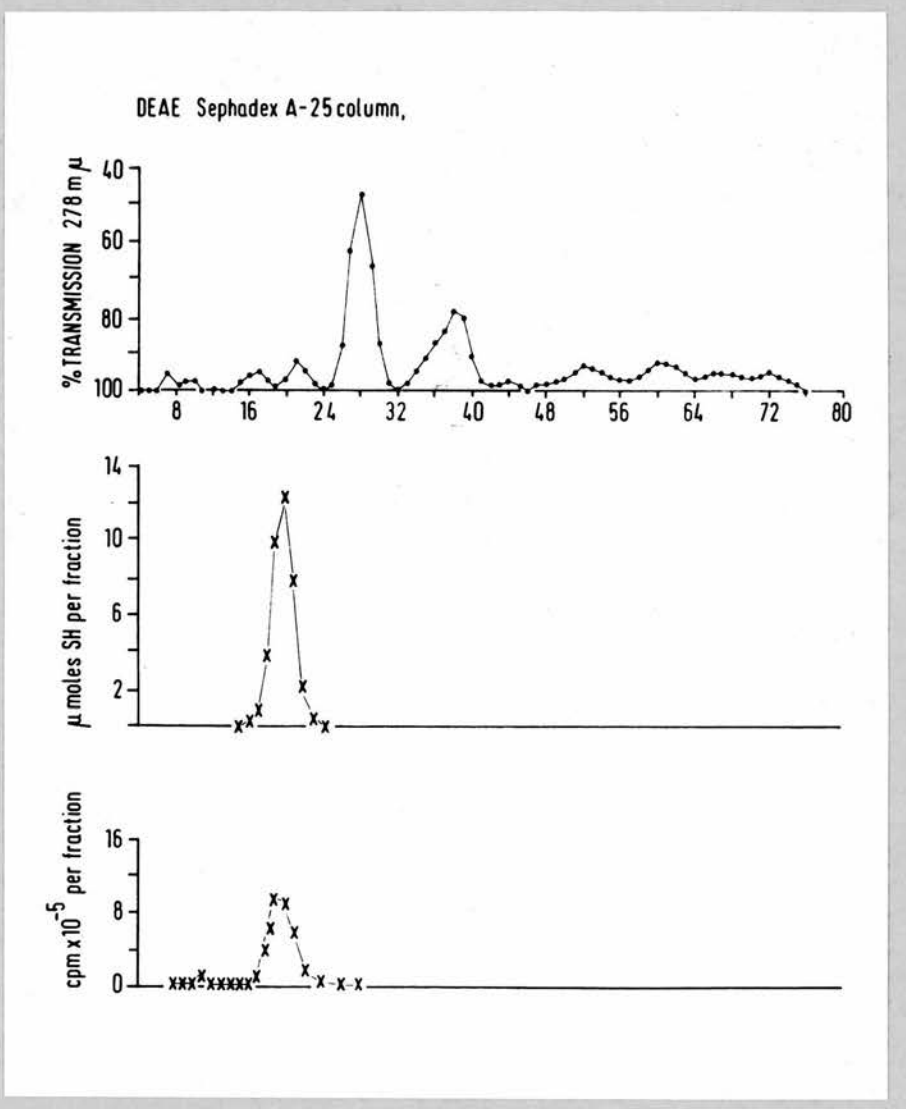

Figure 37. The DEAE-Sephadex A25 chromatography of the thiol fraction from Sephadex G25 chromatography of the heat-stable supernatant of the rat liver.

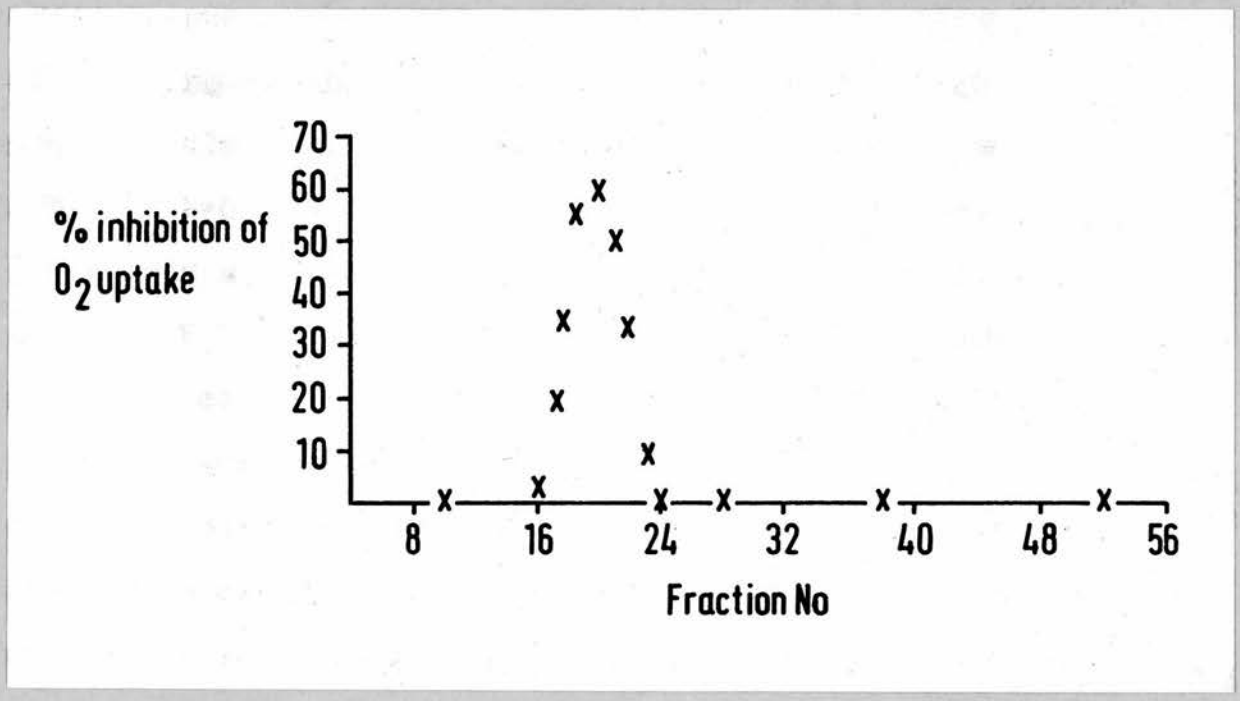

Figure 38. Effect of fractions from DEAE-Sephadex A25 column on oxygen uptake by rat liver microsomes.

H. Further Fractionation of the S.F.

Further chromatography of the thiol fraction from the G-25 column after lyophilization, was carried out by ion exchange chromatography. The column used for the second part of the fractionation was a DEAE Sephadex A25 column, pre-equilibrated with 0·05 M phosphate buffer, pH 7·0. The lyophilized thiol fraction was applied to the column and eluted with the phosphate buffer containing a gradient of KCl from 0 to 0·5 M. The elution profile is shown in Figure 37, and once again a single thiol peak and coincident radioactive peak was obtained. Figure 38 shows the effect of addition of the thiol fractions to the O_2 uptake assay mixture. The peak of inhibitory activity is coincident with the thiol peak, demonstrating that the thiol fractions retain their antilipoperoxidative activity through two column fractionations. This could be taken as an indication that there is only one component in the lipoperoxidation-inhibiting system, as it is unlikely that two dissssociated components would remain together throughout two purification steps. However, measuring the activity of the thiol fraction from DEAE-Sephadex A25 in the inhibition of the production of malonaldehyde from the liver microsomes, shows that the thiol from the DEAE column is less active than the heat-treated supernatant or the G-25 column thiol fraction, when compared on the basis of thiol concentration.

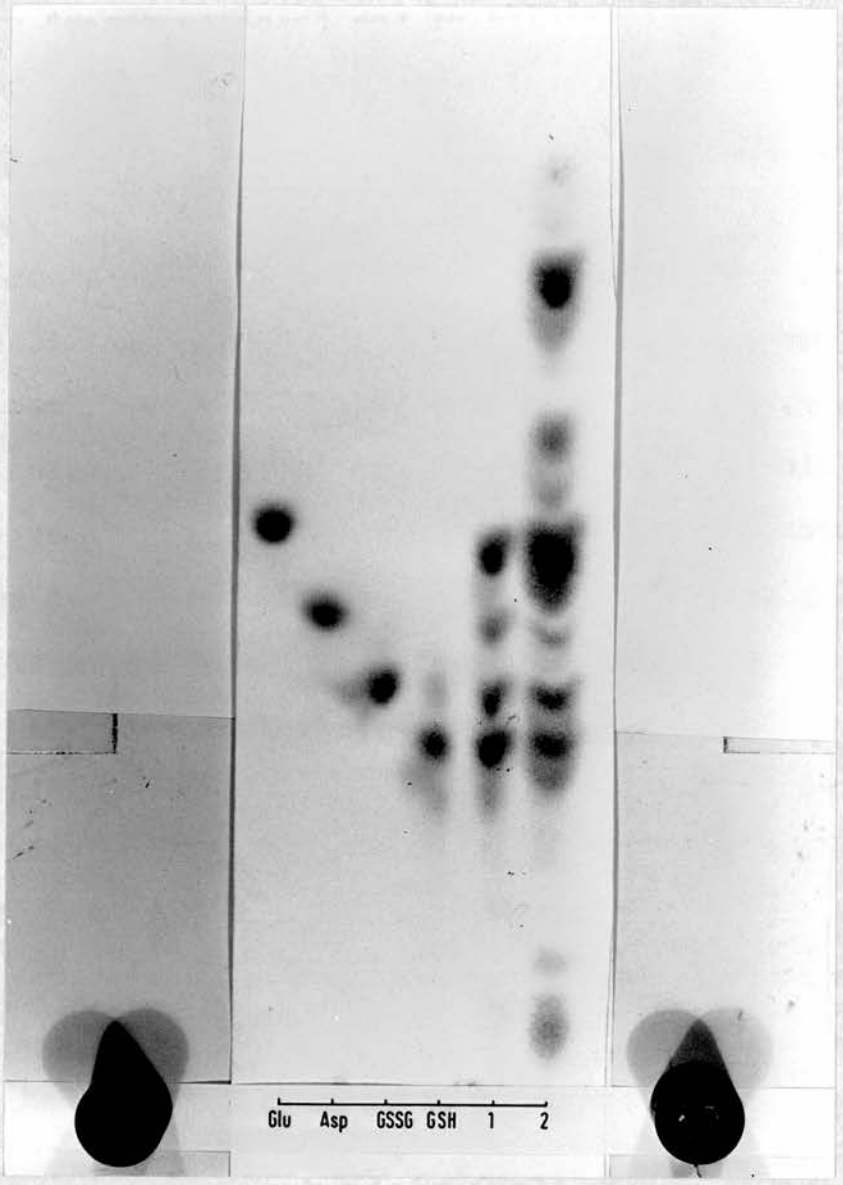

Figure 39. The electrophoresis of the thiol fractions from (1) DEAE-Sephadex and (2) G25 column chromatography as indicated in Figs. 35 & 37, together with standard GSH, GSSG, aspartate and glutamate. (Electrophoresis on Whatmann 3 MM paper, formic acid/acetic acid pH 2·0, 2,000 volts, 2 hours)

I. Identification of the Thiol Component of S.F.

The identification of the thiol component of the S.F. was carried out by electrophoresis of the thiol fraction from the DEAE-Sephadex column. The electrophoresis was carried out on paper at pH 2·0 in a white spirit tank at a voltage of 2,000 v for 2 hours. Four ninhydrin positive spots were obtained after drying the paper and spraying. The thiol was identified by radioactive scanning, and only one major radioactive peak was obtained. Each ninhydrin positive spot was identified by elution from the paper, acid hydrolysis, and aminoacid analysis by paper electrophoresis and they were found to be glutathione (oxidised and reduced) aspartate and glutamate. The electrophoresis of the thiol fraction from the DEAE Sephadex column is shown in Figure 39 along with standard compounds. The radioactivity is confined mainly to the peak identified as glutathione in its reduced form. The identification of the thiol component of the S.F. as reduced glutathione is surprising for a number of reasons. Firstly, the thiol fraction from the G-25 Sephadex chromatography runs at an apparent molecular weight of approximately 2,000 to 3,000. It might be suspected therefore that the thiol compound isolated is a protein of molecular weight 2 - 3,000 which has an amino acid composition of glycine:cysteine:glutamic acid in a ratio 1:1:1. Standard high grade commercial reduced glutathione, however, is excluded from G-25 Sephadex to the same extent as the thiol fraction of the S.F. The molecular sieving properties of G-25 Sephadex must be anomalous for this compound. The unusual properties of

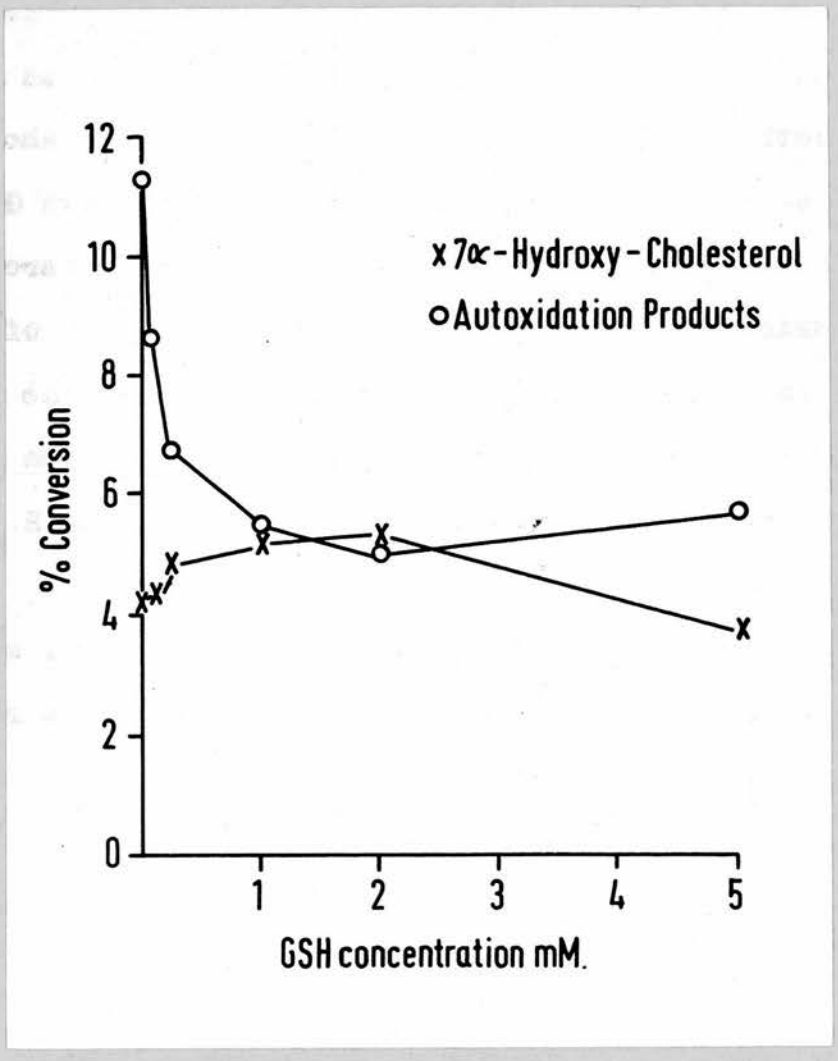

Figure 40. The effect of GSH on cholesterol oxidation.

G25 Sephadex are discussed further in Appendix 1.

Secondly, glutathione is known to be inefficient at inhibiting lipid peroxidation. Wills (57) has shown that GSH has little effect on the oxygen uptake and the malonaldehyde production of liver microsomes. A comparison between reduced glutathione and the S.F., both at physiological concentrations is shown in Table 7. As can be seen, S.F. is far more efficient than GSH in the stimulation of the 7α-hydroxylation of cholesterol, the inhibition of autoxidation of cholesterol, the inhibition of O_2 uptake and the inhibition of production of malonaldehyde by the liver microsomes. This point is further demonstrated by comparison of Figures 40 and 30. These figures show the effect of GSH and S.F. respectively on the cholesterol-7α-hydroxylase assay system. GSH does cause a certain amount of inhibition of the cholesterol autoxidation, but the 7α-hydroxycholesterol:autoxidation products ratio is at no time greater than 1:1 compared with 5:1 for the S.F. at the same concentration.

The conclusions arrived at therefore are that (1) glutathione is the thiol component of the S.F. necessary for its anti-lipoperoxidative activity and (2) there is some other component in the S.F. which modifies the behaviour of the GSH in some way, and causes the strong anti-lipoperoxidative activity found in the S.F.

The conclusion that there is at least one other component in the S.F. apart from GSH is upheld by the fact noted above that the thiol fraction from the DEAE Sephadex column is less effective than the unfractionated S.F. or the thiol fraction from the G25 Sephadex

Table 7.

Comparison of reduced glutathione and S.F. as inhibitors of cholesterol autoxidation, oxygen uptake and malonaldehyde production in the rat liver microsomes under standard conditions.

		No. additions	6 mM GSH	6 mM S.F. Thiol
Cholesterol-7α-Hydroxylase Assay.	% 7α-hydroxy-cholesterol	4·0	4·8	11·2
	% Autoxidation Products	8·8	4·7	1·7
	Autoxidation % of control	100	54	19
Oxygen Uptake	% of control	100	78	17
Malonaldehyde Production	% of control	100	74	9

column at inhibiting lipid peroxidation when compared on the basis of thiol concentration. It is also found that, on storage of the DEAE Sephadex thiol fraction, the inhibitory activity is lost within a few days, whereas the thiol titre goes down only very slowly. The reason for the lower efficiency of the DEAE Sephadex thiol fraction could therefore be either a partial separation of the GSH from the other component(s) of the anti-lipoperoxidative system or inactivation of the other components during the column chromatography. The fraction from the DEAE Sephadex column does however contain the other component(s).

The non-radioactive ninhydrin-positive spots obtained on electrophoresis of the DEAE Sephadex thiol fraction were, as already mentioned, aspartate and glutamate. The oxidised glutathione is probably formed during the application of the thiol to the electrophoresis paper, as the amount present, judged by the ninhydrin colour varies from separation to separation of the same sample, the GSSG-associated radioactive peak also varying in its proportion to the GSH associated radioactive peak. It is not surprising that GSH, glutamate, and aspartate emerge at the same place from an ion exchange column as all have one free amino group and two free carboxyl groups, giving each the same charge at the pH of the column.

Various proportions of oxidised glutathione, aspartic acid and glutamic acid were added to a fixed amount of reduced glutathione to discover if there was any modification of the anti-lipoperoxidative activity of glutathione by the other ninhydrin positive components

of the DEAE Sephadex thiol fraction. The activity of the glutathione remained unaffected by the addition of these components, and so they can probably be ruled out from taking any part in control of lipid peroxidation in the cell.

Spectrophotometry of the DEAE Sephadex thiol fraction shows the presence of UV absorbing material with a peak at 262 nm. This is probably a nucleotide, but has not been characterised.

SUMMARY OF RESULTS

1) The heat stable supernatant of the rat liver contains a powerful anti-lipoperoxidation system.

2) Prolonged heating and dialysis removes the ability to inhibit peroxidation from the heat-treated supernatant.

3) Thiol groups are vital for the activity of the heat-stable supernatant.

4) Fractionation of the heat-stable supernatant by exclusion chromatography yields a single thiol fraction with peroxidation inhibiting activity.

5) Ion exchange chromatography of the thiol fraction from exclusion chromatography yields a single thiol fraction.

6) The thiol is reduced glutathione.

7) Oxidised glutathione, aspartic and glutamic acids, and unidentified U.V. absorbing materials are present along with reduced glutathione in the thiol fraction from DEAE Sephadex chromatography.

8) Other unidentified components are present in the anti-lipoperoxidative system as GSH is not active either by itself, or in the presence of the other identified components.

SECTION 7

THE MODE OF ACTION OF THE HEAT-STABLE ANTI-LIPOPEROXIDATION SYSTEM

A. Introduction

In order to account for the production of malonaldehyde in the peroxidation of unsaturated fatty acids and the variety of products obtained from the autoxidation of cholesterol, the formation of lipid peroxides must be postulated. A scheme showing the production of the autoxidation products of cholesterol from cholesterol-7α-hydroperoxide is shown in Figure 20, and the production of malonyl dialdehyde by peroxidation of an unsaturated fatty acid is shown in Figure 21.

The production of hydroxylated products from P450 dependent mixed function oxidase reactions has been suggested to procede via a peroxide or hydroperoxide intermediate (60), but this has not been confirmed in many instances. The observation that lipid peroxidation reactions require NADPH, and that they can be inhibited by cytochrome c, and other electron acceptors of appropriate redox potential (61) suggests that the peroxidation reactions have at least part of their electron transport pathway in common with the cytochrome P450-dependent microsomal mixed function oxidases. Adding to this the fact that lipid peroxidation reactions apparently do not occur *in vivo* in the normal state and it seems reasonable to suggest that lipid peroxidation reactions are an aberrant form of mixed function oxidation which produce free radicals from peroxides which initiate autocatalytic free radical chain reactions.

The mode of action of the heat-stable supernatant factors (S.F.) could either be to inhibit the production of free radicals or to cause the free radicals produced to break down into harmless relatively inert products such as hydroxides, as suggested by Little et al. (56) and Christophersen (55).

The mechanism whereby the autocatalytic reactions caused by X-irradiation are inhibited by thiol compounds is thought to be as follows

$$2RO_2^{\cdot} + 2R'SH \longrightarrow 2R'S^{\cdot} + 2ROOH$$

$$2R'S^{\cdot} \longrightarrow R'SSR'$$

It is probable that the active form of the thiol is the ionized form, $R'S^-$, which will readily donate an electron to the $RO_2^{\cdot}$ radical. In other words the pK of the thiol group may affect the efficiency of the thiol in inhibiting the peroxidation. The pK of two thiols and the S.F. thiol were therefore measured to discover if there was any validity in this argument.

It is known that there is an enzyme in the rat liver supernatant which will catalyse the conversion of linoleic acid hydroperoxide to a hydroxy acid, at the same time oxidising GSH (55). This reaction could conceivably occur without the enzyme (62) in the heat-treated supernatant, and this possibility was tested.

Finally, the fact that metal ions have a marked effect on peroxidation has already been noted (c.f. p. 53),and metal ions are known to interact with GSH in prevention of hyperbaric oxygen

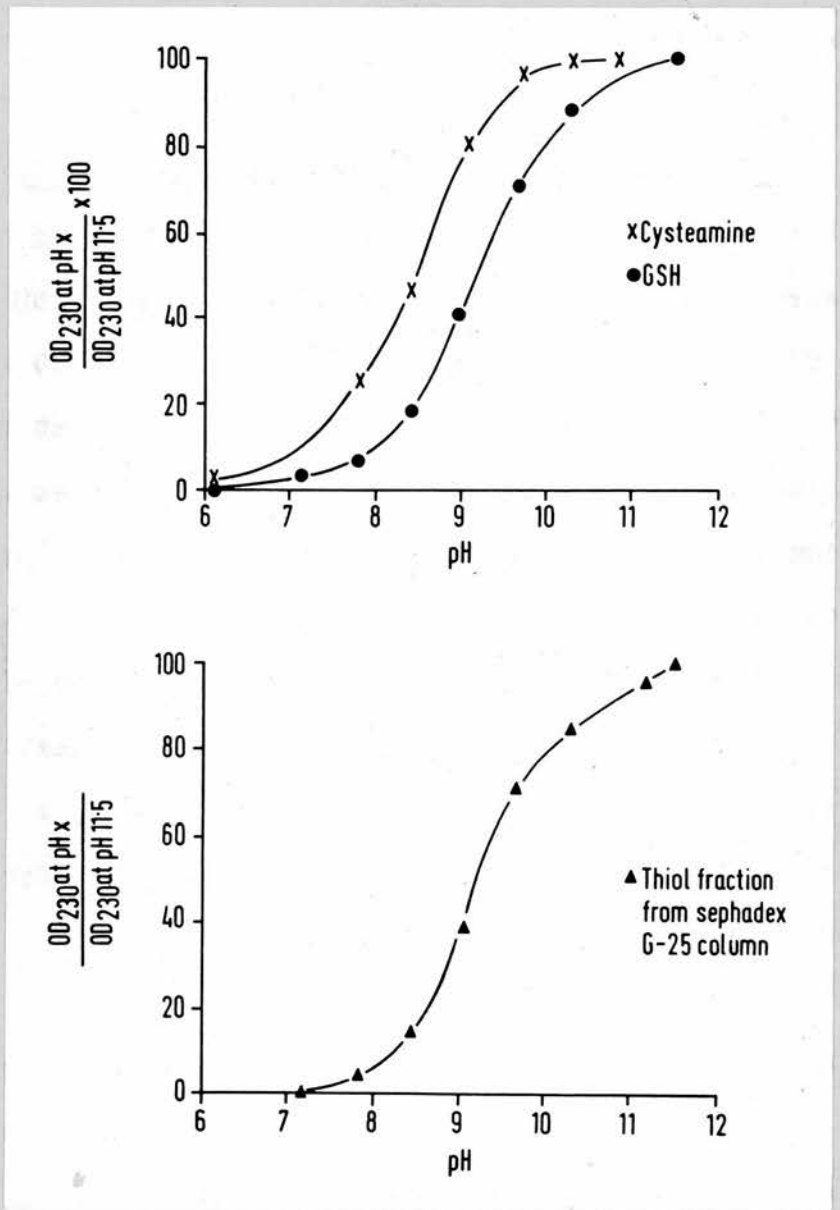

Figure 41. The determination of the pK of GSH and cysteamine (top) and the pK of thiol in heat-treated supernatant of the rat liver. (bottom)

damage (63). The effect of various combinations of GSH and metal ions was investigated.

B. The pK of the S.F. Thiol Groups and Other Thiol Compounds

The pK of a thiol compound can be measured amperometrically, or spectrophotometrically, as has been done in this case. This can be done as the RS^- anion absorbs strongly at 230 nm, whereas the RSH, undissociated form has no absorbance at this wavelength (64). Thus, by measuring the absorbance at 230 nm of a thiol compound at various pH values a titration curve can be obtained and the pK can be found graphically. This is shown in Figure 41 for cysteamine and glutathione. The amount of thiol in the dissociated form is calculated by calculating the ratio of the absorbance at a given pH to the absorbance at pH 11·5 where all the thiol is assumed to be in the ionised form. The pK is equal to the pH when there is 50% of the thiol in the ionised form. The pK of cysteamine therefore is 8·4 and that of glutathione 9·2. These values agree very closely with those found by Benesch et al. (64). It is possible from these two values to postulate that the pK of a thiol does have a bearing on its activity in inhibiting lipoperoxidative reactions, cysteamine being more effective than glutathione and also having a lower pK.

The pKs of the thiol component from the G25 column and of the unfractionated heat-treated supernatant were determined in the same way. The curves were found to be superimposed on each other, the one for the fractionated thiol being shown in Figure 41. As

can be seen the pK as determined by this method is 9·2. There is therefore no difference between the pure GSH and the thiol in the presence of at least one factor which is known to modify its behaviour.

C. The Effect of S.F. on Cholesterol-7α-Hydroperoxide

It is conceivable that the reaction

$$ROOH + 2GSH \longrightarrow ROH + GSSG + H_2O$$

could occur non-enzymatically (62).

In the case of cholesterol-7α-hydroperoxide, this would produce 7α-hydroxycholesterol. Cholesterol-7α-hydroperoxide prepared by photooxygenation in this laboratory by S.A.M. Ali (68), was incubated with GSH and with S.F. for 30 minutes and the products were extracted and separated by TLC. The products were visualised with sodium dodeca-phosphomolybdate spray. Some 7α-hydroxycholesterol was formed, but there was no difference in amounts formed in the incubations containing GSH, the S.F. or the control. This suggests that there is no non-enzymically catalysed reduction of the hydroperoxide occurring with S.F.

D. The Interaction of Glutathione and Trace Metals

Trace metals and glutathione are both known to cause an inhibition of the damage caused by ionizing radiation and by hyperbaric oxygen (65,66,67), *in vivo*. In some cases they are known to potentiate each other (63). As has been pointed out

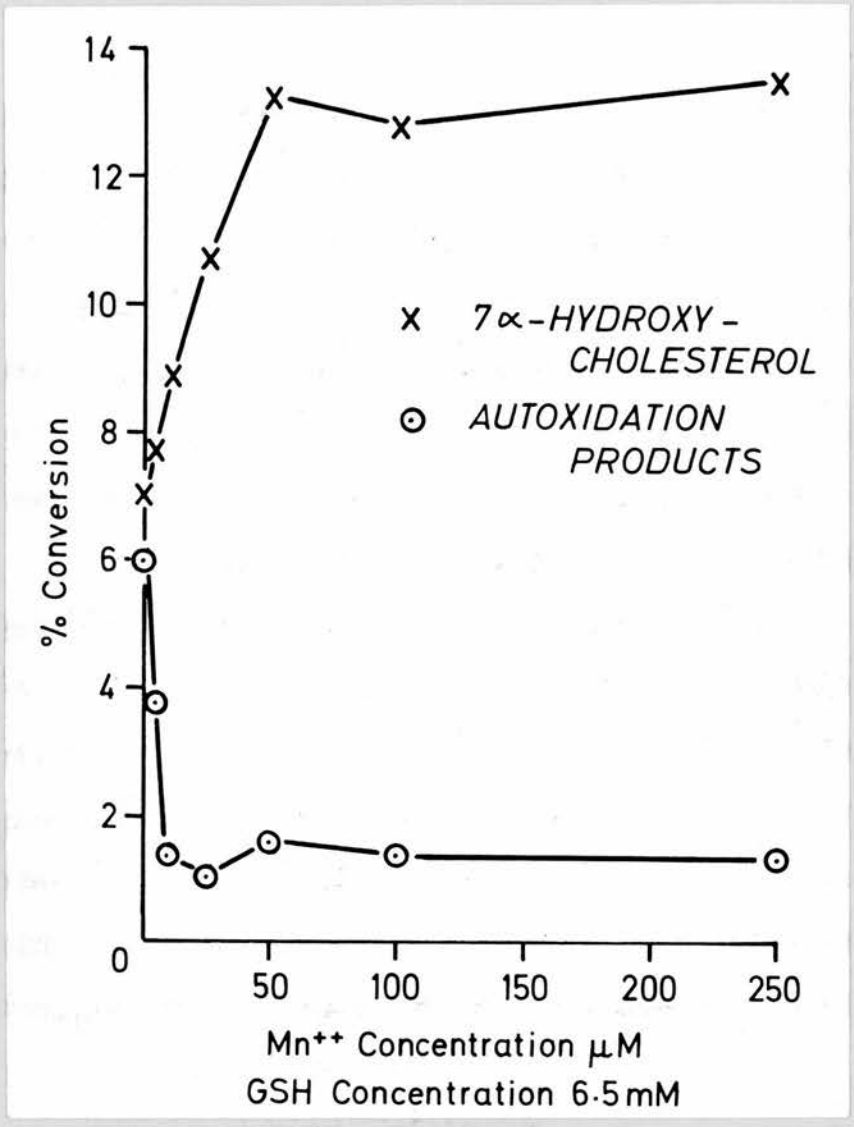

Figure 42. The effect of manganous ions in the presence of 6·5 mM GSH on cholesterol oxidation in liver microsomes.

there are several similarities between the condition of X-ray-induced and hyperbaric oxygen-induced lipid peroxidation, and NADPH supported lipid peroxidation in the microsomes. The effect of some trace metals, and the effect of glutathione on the cholesterol-7α-hydroxylase has already been demonstrated. In view of the facts mentioned it would be interesting to discover if there is any interaction between these two factors in modifying the cholesterol-7α-hydroxylase enzyme system.

A very interesting result is found when varying concentrations of manganous ions are incubated in the presence of 6·5 mM GSH in the cholesterol-7α-hydroxylase assay. As can be seen in Figure 42, the autoxidation of cholesterol is inhibited, and, in addition, the production of 7α-hydroxycholesterol is stimulated as the concentration of manganous ions increases, in sharp contrast to the situation existing in the presence of manganous ions alone (c.f. Figure 29). In other words, the combination of reduced glutathione and manganous ions in low concentration has the same effect on cholesterol-7α-hydroxylation as the S.F. Either of these compounds separately does not have this effect as can be seen in Figures 29 and 40.

It is possible therefore that the factor which modifies the effect of GSH in the S.F. is a metal ion, possibly manganous. The effect of other metal ions in the presence of 6·5 mM GSH on the cholesterol-7α-hydroxylase system is shown in Table 8. (Taken from Boyd et al. (58)). Manganous is the most effective, with cobaltous and chromous also having a similar but less potent effect.

Table 8.

The effect on metallic ions in the presence of 6·5 mM GSH on cholesterol oxidation of rat liver microsomes.

Metallic ion (0·10 mM)	% 7α-hydroxy cholesterol	% Autoxidation products
Mn^{++}	13·5	1·3
Co^{+++}	10·9	1·7
Cr^{++}	6·8	4·1
Mg^{++}	4·1	8·7
Ni^{++}	3·8	5·0
Zn^{++}	3·6	6·0
Fe^{++}	1·1	13·4
No addition	3·9	9·0

The concentration of these ions in the S.F. is of critical importance. The concentration of manganous ions, the most abundant of these ions which are effective, is 1·8 μM in the liver supernatant (69). The concentration of cobaltous ions is a factor of 10^3 smaller (70) and chromous ions are not detected. The manganous ion concentration in S.F. was also measured by emission spectrometry and found to be below the limits of detectability of the instrument, that is a concentration of about 1·0 part per 10^6, which is equivalent to less than 20 μM.

Taking the physiological concentrations of the effective metallic ions and incubating them with 6·5 mM GSH in the cholesterol-7α-hydroxylase system produces no significant effect on the system over and above that produced by the GSH alone. It therefore seems that the metallic ion concentration in the S.F. is too low to exercise the effect seen with higher concentrations of manganous ions, and that the possibility of metallic ions being the potentiating factors of GSH in the S.F. must be rejected.

SUMMARY OF RESULTS

1) The pKs of GSH and cysteamine were compared with that of the thiol and the heat-stable supernatant and that in the thiol fraction from Sephadex G25 chromatography. GSH and the thiol have identical pK values.

2) The S.F. was tested for the ability to reduce cholesterol-7α-hydroperoxide. This was found to be absent.

3) The interaction of GSH and metallic ions, especially manganous was tested. Manganous ions and GSH in appropriate concentrations mimic the action of S.F. on the cholesterol-7α-hydroxylase system, but the physiological concentration of manganous ions and other effective metallic ions is too low for this to be of physiological importance.

SECTION 8

DISCUSSION AND CONCLUSIONS

The 7α-hydroxylation of cholesterol has great importance as the rate limiting step in the pathway of cholesterol catabolism which is most significant quantitatively. The administration of cholestyramine resin is one of the most effective means of lowering the blood cholesterol level (91), and it is found that this treatment also greatly increases the level of cholesterol-7α-hydroxylase in the liver (7). It is likely that the activity of the cholesterol-7α-hydroxylase enzyme has some bearing on the regulation of blood cholesterol levels. It is therefore important to understand the mechanisms whereby the enzyme activity is mediated and its relationship to the other hydroxylase enzymes of the liver.

The cholesterol-7α-hydroxylase is a typical liver microsomal mixed function oxidase enzyme. It has a requirement for NADPH, which cannot be replaced by NADH, or any other donor of reducing equivalents which has been tried. The enzyme also has a requirement for molecular oxygen, and thus comes within the class of enzymes known as 'mixed function oxidases'. As is the case with many other microsomal mixed function oxidases with a requirement for NADPH, the enzyme is inhibited by carbon monoxide, and the inhibition is reversed by light, the most effective wavelength for reversal of inhibition being 450 nm. The enzyme therefore involves the microsomal cytochrome P450 for its activity.

Cytochrome P450 accounts for about one half of the haem of the rat liver microsomes, and has been implicated in the hydroxylation of a very large number of hydrophobic substrates. The cholesterol-7α-hydroxylase is therefore a typical microsomal hydroxylase in this respect. Another point of similarity between the cholesterol-7α-hydroxylase and other hydroxylases of the liver microsomes is the inhibition of the enzyme by cytochrome c. This indicates the involvement of the flavoprotein NADPH-cytochrome c reductase in the action of the enzyme. In these basic respects therefore the cholesterol-7α-hydroxylation is identical to many other hydroxylations which occur in the liver microsomes. It is known that the hydroxylation of a very wide variety of substrates occurs by the action of one or a very few P450 enzymes, and it is possible that the hydroxylation of cholesterol is catalysed by the same enzyme as that which catalyses the hydroxylation of hexobarbital or benzpyrene, for example. It would be very unusual however if the rate of hydroxylation of drugs was controlled by the same factors as the 7α-hydroxylation of cholesterol, which is a very specific reaction and fulfils a different metabolic function. It is in fact found that there is no change in drug hydroxylation on induction by cholestyramine feeding of the cholesterol-7α-hydroxylase which in turn does not change on induction of drug hydroxylation (35). It is also found that the level of cytochrome P450 in the liver microsomes does not increase on induction of the cholesterol-7α-hydroxylase, whereas it increases by as much as 5-fold on induction of the drug hydroxylase.

One possible explanation for this discrepancy is that there is more than one species of cytochrome P450 present in the liver microsomes and that qualitative rather than quantitative changes occur in the total microsomal P450 on induction of the cholesterol-7α-hydroxylase. That more than one subspecies of cytochrome P450 exists in the microsomes has been shown by several groups of workers using a wide variety of techniques. Using tritiated δ-aminolaevulinic acid to label the haem of the cytochrome P450, Levin and Kuntzman showed that the breakdown of P450 occurs biphasically, and that one subspecies has a half-life of 7 hours whereas the second sub-species has a half-life of 48 hours (37). In normal rats the ratio of the fast phase to slow phase components is 4:1, and in methylcholanthrene treated rats the ratio is 1:1 (71). These ratios are very similar to those calculated by the n-octylamine difference spectrum method which has been described in Section 3 (36). Hildebrandt et al. demonstrated that the P450 in microsomes from rats treated with methylcholanthrene was of a different species from that in rats treated with phenobarbital, and equated the two sub-species found with cytochrome P450 in which the haem is in either the high or low spin state (72). More recently, Imai and Siekevitz have demonstrated that the two forms of P450 described by these various groups are in fact a single haemoprotein which can exist in two forms in the microsomal membrane, the haem of the high-spin, slow-phase P450 being held in a more hydrophobic environment than the haem of the low-spin,

fast-phase P450 (73). The high-spin, P450 is thought to be involved in the hydroxylation of hydrophobic substrates such as polycyclic hydrocarbons, and the low-spin P450 in the hydroxylation of more polar drugs such as ethylmorphine and hexobarbital. Spectral examination of the cytochrome P450 in the liver microsomes of cholestyramine-fed rats, by use of the n-octylamine difference spectrum technique reveals that there is no change in the ratio of high- to low-spin P450 on induction of the cholesterol-7α-hydroxylase, thus indicating that a transition between these two forms plays no part in the induction process.

Several groups of workers have shown that substrates which are hydroxylated by cytochrome P450 dependent enzymes cause competitive inhibition of each others hydroxylation. For example, ethylmorphine competitively inhibits the oxidation of laurate and vice versa (25). Cholesterol-7α-hydroxylase is not inhibited by either aminopyrine or aniline; in fact it is stimulated. On the other hand, there is no difference in the rate of aminopyrine demethylation or aniline hydroxylation in the liver microsomes from cholestyramine fed rats, in which cholesterol hydroxylation is proceeding at up to five times the normal rate. If the increased rate of cholesterol-7α-hydroxylation is due to increased availability of cholesterol at the active site of the enzyme, and the active site is the same as that involved in the demethylation of aminopyrine and aniline hydroxylation, then decreased activity of these reactions might be expected due to competition at the active site.

Similarly, Orrenius and Thor have shown mutual competitive inhibition of binding of substrates forming difference spectra with the cytochrome P450 (25). However, there is no detectable difference between the spectral dissociation constants or the magnitude of the spectral changes caused by the binding of aminopyrine or aniline in the microsomes from control or cholestyramine-fed rats.

Thus it appears from this data that the interaction of cholesterol with the cytochrome P450 is not altered in any way by the other substrates of P450 dependent reactions which were tested.

The explanation for these results could be that the 7α-hydroxylation of cholesterol is carried out by a sub-species of cytochrome P450 which is highly specific for the cholesterol molecule, and constitutes a very small fraction only of the total liver microsomal P450. This P450 might have a binding site for the cholesterol molecule which is highly specific, and therefore excludes other substrates of P450-dependent reactions. Increases in this sub-species would be undetectable due to the presence of large amounts of drug-hydroxylating P450. A difficulty with this type of theory is to explain why the cholesterol is not hydroxylated by the non-specific hydroxylase activity of the microsomes. Many other steroids such as testosterone and pregnenolone are substrates of the 'general' hydroxylase (74), testosterone for example being hydroxylated in the 6β, 7α, and 16α positions (16). Cholesterol is only hydroxylated at the 7α position.

This difficulty does not arise with an alternative explanation of the results which does not postulate a minor, highly specific cytochrome P450 for the cholesterol-7α-hydroxylase. It is possible that some component exists which is highly specific for cholesterol and causes binding of cholesterol to the hydroxylation active site in such a way that only the 7α-hydroxylation can take place. The 7α-hydroxylation could then occur at the 'general' hydroxylase, and the increase in activity on induction of the cholesterol-7α-hydroxylase by cholestyramine feeding could be accounted for by an increase in the cholesterol binding factor. This hypothesis gives an adequate explanation of the lack of changes in the spectral or metabolic characteristics of the liver microsomes on induction of the cholesterol-7α-hydroxylase, but there is no positive evidence to support it.

A part of this investigation was involved in attempts to solubilize, fractionate and purify the components of the cholesterol-7α-hydroxylase, in order to throw some light on the above problem. The assumption was made that the cholesterol-7α-hydroxylase had properties similar to those of the drug hydroxylase enzymes present in the microsomes. This assumption was made as many factors which inactivate the drug hydroxylase by causing conversion of cytochrome P450 to the inactive cytochrome P420 form also inactivate the cholesterol-7α-hydroxylase. Any process of solubilization which caused the conversion of cytochrome P450 to P420 was therefore abandoned. All the difficulties encountered during the solubilization of the drug hydroxylase were encountered

in this study, plus a few more due to the inhibition of the enzyme activity by some of the solubilizing agents.

The necessity for an absolute measurement of the activity of the cholesterol-7α-hydroxylase enzyme also became apparent. In all the studies of the intact microsomes it is assumed that the radioactive tracer cholesterol added to the incubation medium equilibrates rapidly with the non-radioactive endogenous cholesterol. That this is the case is borne out by the fact that the rate of incorporation of radioactive cholesterol into 7α-hydroxycholesterol is linear from the time of addition of the radioactive cholesterol. If equilibration was delayed or slow, a lag or an acceleration of incorporation would be expected. When studies are done on solubilized preparations of the microsomes or on microsomes which have been treated with various reagents apparent increases in the activity of the cholesterol-7α-hydroxylase occur. For example, on preparation of an acetone powder from microsomes which have an activity of 7% of the cholesterol converted to 7α-hydroxycholesterol per hour, the activity increases to 24% conversion per hour. This increase in activity is not due to an activation or purification of the cholesterol-7α-hydroxylase, but to a decrease in the amount of cholesterol present in the preparation. There is therefore less cholesterol present to equilibrate with the added tracer. It is impossible with a tracer assay system to carry out comparative quantitative measurements of cholesterol-7α-hydroxylase activity in different preparations. An absolute assay method which measures the mass of 7α-hydroxycholesterol produced is necessary. The

activities of various solubilized and fractionated preparations in this investigation are therefore of a qualitative nature.

Little new light was thrown on the nature of the cholesterol-7α-hydroxylase enzyme by this investigation, although many techniques for solubilisation and fractionation of the enzyme were attempted. Techniques which have been developed in the study of other membrane bound enzyme systems were applied to the cholesterol-7α-hydroxylase enzyme system. Soluble preparations of the cholesterol-7α-hydroxylase were obtained by use of high-molarity buffers, and ammonium sulphate fractionation of cholate-solubilised microsomes. These preparations were not amenable to fractionation. A solubilised preparation of the enzyme was also obtained by application of the Lu and Coon procedure (26). Attempts at fractionation of the enzyme by this method resulted in active components, but no enzyme activity was recovered. Modifications of this method probably hold the best hope for future fractionation of the cholesterol-7α-hydroxylase.

One interesting result concerns the interaction between the cytochrome P450 and the NADPH-cytochrome P450 reductase. In two cases, (See sections 4C & D), an active NADPH-cytochrome c reductase and an undegraded P450 preparation were obtained, but no NADPH-P450 reductase activity was present. In the case of lipid extraction, this loss of interaction is thought to be due to the loss of essential phospholipid material, and in the case of proteolytic digestion technique, the loss of interaction is probably due to lack of interaction between the reductase and the P450. These

two results show the importance of orientation and environment of the components of the enzyme for effective electron transport.

The problem set by the induction and P450 studies was not resolved by the solubilisation studies. The close relationship established by the work already discussed between the drug hydroxylation and cholesterol hydroxylation was maintained.

The mechanism of hydroxylation by P450 dependent mixed function oxidase enzymes has been studied in three hydroxylating systems for the most part, namely, the adrenal cortex mitochondrion, the liver microsomes and the camphor oxidase system of Pseudomonas putida. Of these three, the bacterial system has been extensively purified, and found to consist of three components, a flavoprotein, an iron-sulphur protein, and a cytochrome P450 (75). The sequence of events at the active site of the enzyme complex, which is part of the P450 molecule, has been worked out in some detail (76). The 11β-hydroxylation of deoxycorticosterone and the side-chain cleavage of cholesterol are both P450 dependent reactions occurring in the adrenal cortex mitochondrion, and work on the purification of the components of these reactions is more advanced than in any other mammalian system. Here again, three components of the electron transport chain are present, a flavoprotein, an iron-sulphur protein, and a cytochrome P450 (77). It appears that a separate cytochrome is present for each of the two oxidations mentioned above (78). The sequence of events at the P450 molecule has been worked out, but in less detail than the P. putida system. The

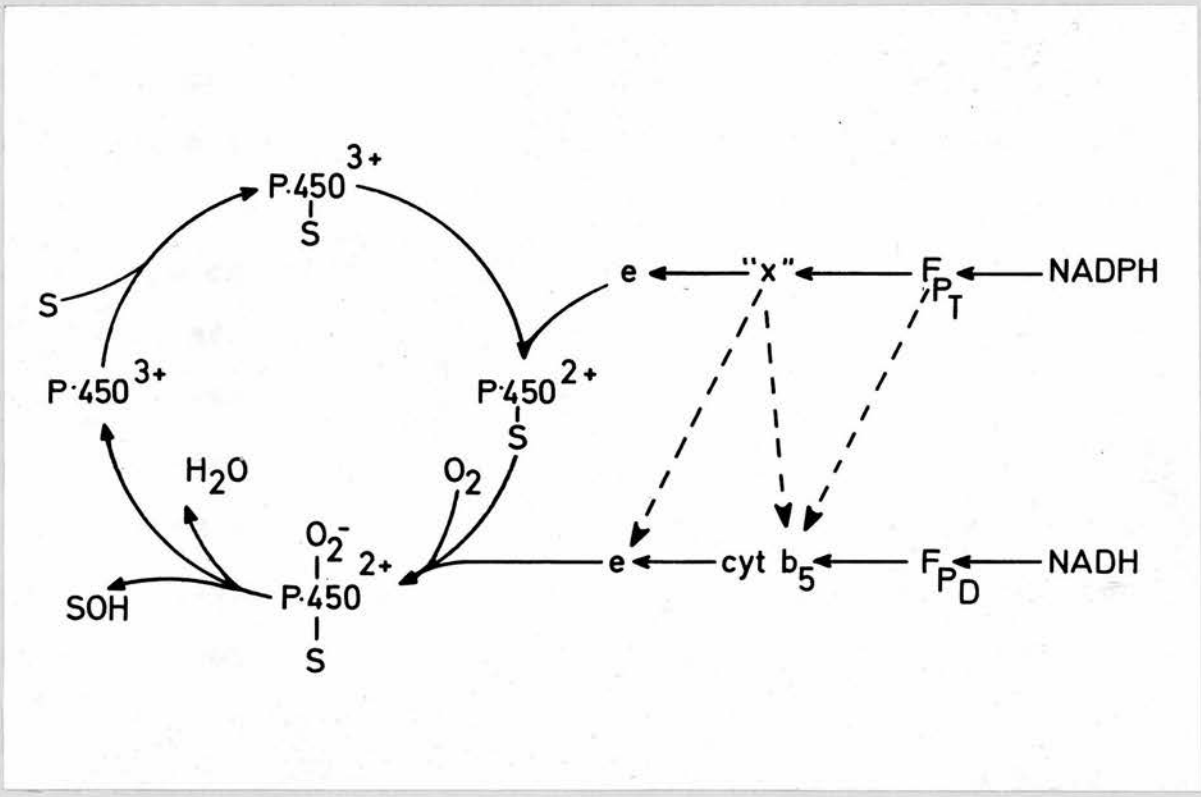

Figure 43. The postulated sequence of events during hydroxylation reactions in the liver microsomes. ($P450^{3+}$ and $P450^{2+}$ are oxidised and reduced cytochrome P450, S is substrate, f_{p_T} and f_{p_D} are reductases specific for NADPH and NADH, 'X' is a hypothetical electron carrier. (Taken from Estabrook et al. (79)).

liver microsomal drug hydroxylase system, being so difficult to solubilize, has been the subject of fewer mechanistic studies. Unlike the other two systems described, it appears to consist of only two electron transport components, a flavoprotein, and the cytochrome P450. Figure 43 shows the sequence of events which are thought to occur at the P450 active centre. This system is that worked out by Estabrook et al. (79) for the drug hydroxylase of the liver microsomes. The sequence of events in all three systems appears to be essentially the same, however, involving binding of the substrate to the oxidised P450, one electron reduction, binding of oxygen, a second electron reduction, followed by release of the hydroxylated substrate and water. In the camphor oxidase system and the 11β-hydroxylase of deoxycorticosterone, there is an absolute requirement for the iron-sulphur protein for the second electron reduction.

In the liver microsomal system, it has been suggested that the cytochrome b_5 may donate the second electron, but is not obligatory (19). F_{p_T} and F_{p_D} are the NADPH and NADH oxidases respectively, and 'X' is a postulated electron transport component suggested by several groups of workers to explain various experimental results (19,80). The exact mechanism whereby the substrate and oxygen interact to form the hydroxylated product and water is unknown, but several lines of evidence point to the formation of P450 (Fe^{2+},O-O$^-$, substrate) complex which breaks down to give the hydroxylated product and water. The possible formation of peroxide intermediates is supported by the isolation of fluorene

and tetralin hydroperoxides during the P450-dependent hydroxylation of these compounds in rat liver (60,81). Cholesterol-20α-hydroperoxide seems a likely intermediate in the cholesterol side-chain cleavage reaction of the adrenal cortex mitochondria (82). Cytochrome P450 has been shown to cause the NADPH dependent reduction of several steroid hydroperoxides (83). It therefore seems possible that cholesterol-7α-hydroperoxide, which Mitton et al. have shown to be a possible precursor of cholesterol autoxidation products (5), is formed by reaction of cholesterol with oxygen at the P450 site.

The very close connection between the lipid peroxidation reactions and the P450 dependent hydroxylations of the liver microsomes has been pointed out on several occasions (51,61). Both processes are specific for NADPH and involve electron transport via the NADPH-cytochrome c reductase flavoprotein. Addition of substrates of P450-dependent hydroxylations causes inhibition of lipid peroxidation. $NADP^+$, which is a competitive inhibitor of NADPH-linked hydroxylations also competitively inhibits lipid peroxidation (61).

The finding that cholesterol is a substrate of cytochrome P450-dependent hydroxylation and also undergoes peroxidation reactions in the liver microsomes has strengthened this connection. The results given in Section 5 show that a 'switch' occurs between production of cholesterol autoxidation products and 7α-hydroxycholesterol on increasing the concentration of certain compounds, notably cysteamine, EDTA and the heat-treated 105,000 g supernatant.

At the same time these compounds inhibit the peroxidation of lipids as measured by malonaldehyde production. All the compounds so far tested which stimulate or inhibit lipid peroxidation have a parallel effect on cholesterol autoxidation. Peroxidation reactions of this type are known to be autocatalytic after initiation, as they proceed by a free-radical chain reaction. In the case of cholesterol autoxidation and lipid peroxidation in the liver microsomes under the conditions of study, initiation of the autocatalytic free-radical reaction is enzymic and dependent on the presence of NADPH. Lipid peroxidation reactions in vivo can be initiated by ultraviolet and other ionizing radiation, hyperbaric oxygen, and introduction of foreign compounds such as carbon tetrachloride (52,53). Lipid peroxidation in these situations can be inhibited by the same agents as microsomal lipid peroxidation, namely, thiol compounds such as cysteamine, EDTA, manganous and other metal ions, and antioxidants such as diphenyl-p-phenylene-diamine.

The site of the reaction at the molecular level between oxygen and unsaturated fatty acids and cholesterol which produces peroxidation products is unknown. It is unlikely to be at the P450 molecule, as lipid peroxidation reactions are not inhibited by carbon monoxide, even though there is some evidence that peroxides are produced during the course of hydroxylation reactions. In addition to the involvement of the NADPH-cytochrome c reductase flavoprotein, there is very good evidence that ferrous iron in a chelated form is necessary for the generation of lipid peroxides (84).

The physiological chelator of the iron in the microsomes is not known, some authors suggesting a non-haem iron protein (factor 'X' of the scheme in Figure 43), and others suggesting nucleotide diphosphate chelates of iron, the most active of these in promoting lipid peroxidation being ADP-ferrous iron complex (28). Mitton et al. have shown that the production of cholesterol autoxidation products is stimulated by the presence of ADP and ferrous ions (5). Oxygen is activated by the ferrous iron, possibly by formation of an iron peroxide free radical (61), which in turn reacts with cholesterol or unsaturated fatty acids to produce a lipid peroxide free radical, the production of peroxides then becoming autocatalytic, as follows.

$$\begin{aligned} RO_2^{\cdot} + RH &\longrightarrow ROOH + R^{\cdot} \\ R^{\cdot} + O_2 &\longrightarrow RO_2^{\cdot} \end{aligned} \qquad (85)$$

That the reaction does proceed autocatalytically can be seen from the accelerating rate of oxygen consumption on initiation of microsomal lipid peroxidation by addition of NADPH.

The seven position of the cholesterol molecule is the most reactive in free radical reactions and therefore the cholesterol-7α-hydroperoxide is formed by the mechanism shown above. This compound can then dehydrate to the 7-keto compound or be reduced to the 7α-hydroxy compound. The 7-ketocholesterol is reduced in the microsomes to 7β-hydroxycholesterol. The formation of cholestan-3,5,6,-triol from cholesterol by autoxidation is less clear but may proceed via a 5-, 6-, oxide. These reactions are shown in

Figure 20. It seems clear from both experimental and theoretical considerations that the formation of unsaturated fatty acid peroxides and hence malonaldehyde, and the formation of cholesterol autoxidation products are two manifestations of non-physiological free radical oxidation reactions in the liver microsomes.

Having established this point, it became of great interest to ascertain how the heat-stable portion of the liver supernatant inhibits the production of cholesterol autoxidation products, as was shown by Mitton et al, and the production of lipid peroxides, as shown in this study.

The results of the search for the identity of the peroxidation inhibiting system are in Section 6. The initial search for the 'S.F.' revealed that it was stable to heating for short periods at 95°, but was inactivated by longer periods of heating. It was not extracted by lipid solvents, and could be removed from the heat-treated supernatant by dialysis, thus indicating that it was a low molecular weight, non-lipid component of the supernatant. Experiments with pCMB showed that the bulk of the supernatant thiol was necessary for the anti-lipoperoxidative action of the supernatant, and chromatography revealed that the thiol was reduced glutathione. Thiols are well known to be antioxidants, and have been used to inhibit damage caused by free radical reactions initiated by irradiation and hyperbaric oxygen <u>in vivo</u>. (96). In Section 6, it is shown that GSH is less effective than heat-treated supernatant at inhibition of NADPH-stimulated peroxidation reactions <u>in vitro</u>, when compared on the basis of thiol concentration. It therefore

appears that, although GSH is the active thiol component of the S.F. *in vivo*, some other component is present which modifies the behaviour of GSH, or the GSH is modified itself in some way which causes an increase in anti-lipoperoxidative activity.

The ability of thiols to inhibit free radical oxidation reactions is due to their acting as hydrogen donors, e.g.

$$2GSH + 2RO_2^{\cdot} \longrightarrow 2GS^{\cdot} + 2RO_2H$$
$$2GS^{\cdot} \longrightarrow GSSG \qquad (62)$$

thus breaking the propagation of free radicals. It follows that the redox potential and therefore the pK of a thiol may have an important bearing on its antioxidant activity. The pK of the thiol in the heat-treated supernatant, in its active antioxidant form, was not different from that of pure GSH. This result does not exclude the possibility that the pK of a small proportion of the GSH was altered in the S.F., as the spectrophotometric method used would not detect a change of this type. The pK of cysteamine, the most effective of the antioxidant thiols was lower than that of GSH, which is far less effective. It therefore seems that the pK may have some bearing on the antioxidant activity of a thiol but the evidence for this proposal was not upheld by the findings with the S.F. thiol.

The results of incubating the S.F. with cholesterol-7α-hydroperoxide demonstrate that the possible reaction

$$ROOH + 2GSH \longrightarrow ROH + GSSG + H_2O$$

does not occur in the supernatant. The removal of cholesterol-7α-hydroperoxide is not a vital reaction for the inhibition of the free radical peroxidation reactions of the liver microsomes as the cholesterol-7α-hydroperoxide is a relatively stable hydroperoxide which is rapidly reduced by the liver microsomes to 7α-hydroxy-cholesterol (5), in the presence of NADPH.

Although certain metal ions, especially manganous ions, in the presence of GSH have an activity identical to that of the S.F., the participation of these ions in the inhibition of peroxidation reactions was thought to be unlikely, due to the low concentrations of these ions present in the liver supernatant. Manganous and cobaltous ions catalyse the decomposition of hydrogen peroxide, and glutathione greatly increases the catalytic activity of either of these metal ions. Mixtures of GSH and cobaltous ions have also been shown to protect cell membranes from oxidative degradation (63).

There is a second possible mechanism whereby the thiol of the liver supernatant may allow the cholesterol-7α-hydroxylase to proceed without interference.

The cytochrome P450 has been shown to be very sensitive to thiol-binding reagents such as pCMB, being rapidly converted to the inactive P420 form (9,86). Scholan has shown that the cholesterol-7α-hydroxylase is also very sensitive to the presence of pCMB, and is rapidly inactivated (35). It is therefore necessary that those thiol groups which are needed for the activity of the enzyme remain intact. The supernatant thiol could have the dual role of inhibiting peroxide formation and maintaining the thiol of

the P450 in the reduced state (87). Lipid peroxides can cause inactivation of enzymes by oxidation of thiol groups and by binding to ε-amino groups of lysine residues (88). Production of very small amounts of lipid peroxides in the microsomes could therefore, by virtue of their proximity to the P450, inactivate the P450 at concentrations of peroxidation products too low to measure. This could explain the apparent activation of the cholesterol-7α-hydroxylase enzyme by cysteamine and the heat-stable supernatant at concentrations which cause inhibition of production of peroxidation products (See Figures 23 & 30). The stimulation of cholesterol-7α-hydroxylase by EDTA could be explained in the same terms, as EDTA is known to chelate heavy metal ions which inactivate thiol groups (89).

The supernatant glutathione, in conjunction with other unknown factors, therefore, appears to have a dual role; protection of the substrate of the cholesterol-7α-hydroxylase enzyme from aberrant autoxidation reactions, and protection of the cholesterol-7α-hydroxylase enzyme from inactivation.

In conclusion, this work has been involved with the oxidation of cholesterol, both physiological and non-physiological in the liver microsomes. The similarities between the autoxidation of cholesterol and the peroxidation reactions of the microsomal lipids lead to the conclusion that the two are essentially the same, and that they are inhibited by the same factors <u>in vivo</u>. The enzyme responsible for the physiological hydroxylation of cholesterol bears many resemblances to those which carry out the hydroxylations

of numerous other lipophilic compounds in the liver microsomes. However, the control of the level of the enzyme for cholesterol remains different from the control of the level of the other hydroxylases.

Future study of this enzyme will throw light on both an important step in cholesterol catabolism and on the mode of action and relationship of liver microsomal hydroxylases.

APPENDIX 1

THE UNUSUAL BEHAVIOUR OF LOW MOLECULAR WEIGHT COMPOUNDS ON G25 SEPHADEX.

It was noticed during the use of Sephadex G25 columns that a compound identified unequivocally as glutathione in its reduced form (M.W. 307) was being partially excluded by the dextran filtration gel. The Sephadex G25 is, according to the manufacturer, to be used for separation of materials of molecular weight 5,000 to 500.

According to Andrews (90), a convenient method for the measurement of the molecular weight of a compound is to use a suitable exclusion chromatography column previously standardised with compounds of known molecular weight, as follows:-

The void volume of the column is determined using a high molecular weight compound such as dextran blue (v_o)

The total volume of the column is determined by measurement (v_t)

The elution volume of the compound is measured (v_e)

The partial exclusion coefficient is determined

$$K_{av} = \frac{v_e - v_o}{v_t - v_o}$$

K_{av} is equal to zero if the compound is completely excluded by the gel, and equal to one if the compound is completely unexcluded by the gel. The K_{av} determined for GSH on several occasions was found to be 0·26.

By drawing a graph of K_{av} against $\log_{10}$ (molecular weight) for a number of standard compounds a straight line is obtained with K_{av} values from 0·2 up to 0·8. By using such a calibration curve the molecular weight given for GSH is approximately 3,500, which is not even within an order of magnitude of the correct result.

Other low molecular weight compounds were run on G25 columns and the results obtained were as shown in the following table.

Compound	Molecular Weight	K_{av}
Sucrose	342	0·30
Cl^- (from NaCl)	35·5	0·33
Cyanocobalamin	1342	0·28
Glutathione	307	0·26

It appears therefore that G25 Sephadex when used under the conditions described does not act as a molecular sieve in the expected sense, as several compounds of a wide variety of molecular weight run with very similar K_{av} values. The conditions used were of very low ionic strength in order to allow concentration of the products of chromatography without interference from large quantities of salt.

G25 columns are used by many workers to desalt preparations of proteins. It would appear advisable from these findings to avoid the use of G25 in this manner for any proteins with a molecular weight of less than 5,000. Any compound with a molecular weight of less than 5,000 will be partially included in the dextran gel and will probably not be completely separated from the salt.

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The Cofactor Requirement for the Rat Liver Microsomal Cholesterol 7α-Hydroxylase Enzyme System

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The aerobic incubation of [4-^{14}C]cholesterol and NADPH with 18000*g* (mitochondria-free) supernatant from rat liver homogenized in 154mM-potassium chloride gives rise to a single radioactive product, cholest-5-ene-3β,7α-diol or 7α-hydroxycholesterol. Incubation of the 105000*g* microsomal pellet under the same conditions gives rise to a spectrum of radioactive 'autoxidation' products of cholesterol, the formation of which can be inhibited by the 105000*g* supernatant.

This anti-autoxidative activity is retained when the 105000*g* supernatant is heated at 95°C for 10min, cooled and centrifuged to remove denatured protein. The cofactor present in this supernatant is diffusible on dialysis, labile to alkali and labile to prolonged heating at 95°C. This activity can be mimicked by certain thiols such as 2-mercaptoethylamine.

The conditions under which cholesterol is autoxidized (Mitton, Scholan & Boyd, 1971) are identical with those in which lipid peroxidation occurs (Ernster & Nordebrand, 1967; Wills, 1969) as measured by oxygen uptake and malonaldehyde production. Heat-treated supernatant will inhibit lipid peroxidation measured by these criteria. The inhibition of cholesterol autoxidation and lipoperoxidation by the supernatant factor(s) is removed by pretreating the supernatant with *p*-chloromercuribenzoate, thus indicating that thiol groups are necessary. Chromatography of freeze-dried heat-treated supernatant on Sephadex G-25 indicated the presence of one main thiol fraction. This fraction inhibits lipid peroxidation. Further chromatography of this thiol fraction on DEAE-Sephadex A-25 gives a single thiol peak with anti-lipoperoxidation activity. This fraction has been examined by electrophoresis and shown to contain GSH, GSSG, aspartic acid, glutamic acid and u.v.-absorbing material. GSH alone or in combination with glutamic acid and aspartic acid does not have any anti-autoxidative activity.

This suggests that GSH is one component of the cofactor involved in the efficient microsomal cholesterol 7α-hydroxylase system. Low concentrations of Mn^{2+} ions in the presence of GSH can imitate the activity of the heat-stable supernatant factor. However, the additional physiological components of heat-stable supernatant have still to be identified.

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